“ Charakterisierung der T-Zell-Antwort auf eine intestinale Nematodeninfektion ”

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1 Zusammenfassung

Parasitische Nematoden beeinflussen gezielt die Abwehrreaktionen ihres Wirtes. Dies wird besonders während der chronischen Infektionsphase durch eine herabregulierte T-Zell-Antwort auf Parasitenantigene und andere Stimuli ersichtlich.

In dieser Arbeit wurde die T-Zell-Antwort gegen einen intestinalen Nematoden untersucht. Mäuse wurden mit dem Trichostrongyliden Heligmosomoides polygyrus infiziert und in der Folge Effektor- sowie regulatorische T-Zellen (Tregs) untersucht. Subpopulationen von CD4+ T-Zellen wurden aus chronisch infizierten Mäusen isoliert und in naive Empfänger transferiert, welche nachfolgend infiziert wurden. Dabei zeigte sich, dass der Transfer von CD4+ Effektor-T-Zellen zu einer verminderten Wurmlast in den Empfängertieren führte, diese Zellen also einen partiellen Schutz gegen die Primärinfektion vermitteln. Der gleichzeitige Transfer von Tregs beeinflusste diesen Effekt nicht. Tregs allein zeigten keinerlei Einfluss auf die Wurmlast der Empfänger.

Die Protektion durch Transfer von Effektor-T-Zellen kann vermutlich auf eine kleine Antigen-spezifische Population von CD4+ Zellen zurückgeführt werden. Diese Zellen wurden durch die Expression von CD40-L (CD154) nach Restimulation mit Parasitenantigen in vitro charakterisiert und enthielten einen Großteil der Zytokinproduzenten unter den CD4+ Zellen. Während diese Effektorzellen ein deutliches Th2-Zytokinprofil durch Produktion von Interleukin-4 (IL-4) und IL-13 zeigten, reagierte eine Treg-Subpopulation mit der Sekretion hoher Mengen von IL-10 auf Antigenstimulation. Diese Tregs waren durch Expression des Integrins αE(CD103)$\beta 7$ sowie CD25 und Foxp3 charakterisiert und vermittelten in vitro die stärkste Suppression anderer T-Zellen, wenn sie aus chronisch infizierten Mäusen isoliert wurden. Durch Untersuchung der zellulären Zusammensetzung von mesenterialen Lymphknoten und Milz konnte gezeigt werden, dass die Frequenz solcher regulatorischer Zellen im Verlauf der Infektion dauerhaft und überproportional zunimmt. Im Gegensatz dazu wurde am Infektionsort nur eine vorübergehende Akkumulation von Tregs (Foxp3+) während der akuten Phase der Infektion nachgewiesen. Diese Ergebnisse zeigen den Einfluss einer intestinalen Nematodeninfektion auf die Aktivität von Tregs und das Potential parasitenspezifischer CD4+ Effektor-Zellen zur Vermittlung von Schutz gegen die Infektion.

Ein weiteres Projekt dieser Arbeit wahr die Verabreichung eines immunmodulatorischen Parasitenproteins, des Filariencystatins Av17, in einem Mausmodell entzündlicher Darmerkrankungen. In Mäusen wurde eine kolitisartige Entzündung durch eine Chemikalie im Trinkwasser induziert. Die regelmäßige Verabreichung von rekombinant exprimiertem Cystatin verminderte die Entzündungsreaktion signifikant. Damit konnte in dieser Arbeit gezeigt werden. dass Entzündungsreaktionen, die nicht durch den Parasiten selbst hervorgerufen werden, durch die Applikation einer einzelnen Parasitenkomponente unterdrückt werden können.

Nematoden, Treg, Th2, Darm, Immunmodulation, Kolitis

1 Summary

Parasitic nematodes specifically modulate the immune response of their hosts. A cellular hyperreactivity, especially during the chronic phase of infection, is a distinct finding of such infections.

The T cell response against an intestinal nematode was analyzed in this work. Mice were infected with the trichostrongylid Heligmosomoides polygyrus and surveyed for changes concerning effector and regulatory T cells (Tregs). Subpopulations of CD4+ T cells were isolated from chronically infected mice and adoptively transferred to naive recipients, which were subsequently infected. The Transfer of CD4+ effector cells conferred partial protection, seen as decreased worm burdens in recipients. This effect was unimpaired by simultaneous transfer of Tregs. The transfer of purified Tregs alone showed no effect on worm burdens.

The protection by transfer of effector T cells was probably due to a small parasite-specific population, which was characterized by the expression of CD40-L (CD154) after antigen-restimulation. The CD154+ population contained high frequencies of cells reacting with production of the Th2 key cytokines interleukin-4 (IL-4) and IL-13. On the other hand, a subpopulation of Tregs secreted high amounts of IL-10 in response to the antigen. These Tregs were characterized by the expression of the integrin αE(CD103)$\beta 7$, as well as CD25 and Foxp3. They showed a peculiar strong suppressive efficacy on the proliferation of other T cells, especially when derived from chronically infected donors. Analyzing the cellular composition of mesenteric lymph nodes and spleens in response revealed a lasting and over-proportional increase in frequencies of these Tregs. In clear contrast, only a transient increase of Foxp3-expressing Tregs was detected at the site of infection during the acute phase. These results point out the changes Treg activity during an intestinal nematode infection and show the potential of CD4+ effector cells in mediating protection against infection.

A second project of this work was the application of an immunomodulatory parasite protein, the filarial cystatin Av17, in a mouse model of inflammatory bowel disease. Mice developed an inflammatory response to a chemical applied in the drinking water. The repeated application of recombinantly expressed cystatin significantly diminished the inflammatory response.

Hence, this work showed the potential of a single parasite component in suppressing inflammatory processes not caused by the parasite itself.

Nematode, Treg, Th2, intestine, immunomodulation, colitis

2 Introduction

2.1 Intestinal worm infections

Helminth infections are widespread in developing countries. More than 2 billion people are infected with parasitic helminths (251), whereby gastrointestinal (GI) nematodes are the most prevalent pathogens among worm infections. Infections with GI nematodes such as *Trichuris trichiura*, *Ancylostoma duodenale* and *Necator americanus* tend to be chronic. A feature of such infections in humans is that some individuals show persistent susceptibility to reinfection after drug cure. Although GI nematode infections are generally not fatal, they lead to high rates of morbidity. In case of severe infections, this may manifest as sustained anaemia, malnourishment, growth retardation and impaired cognitive functions (163). Severe infections may thereby become fatal, especially in children. Of note, GI nematodes may also increase the risk to suffer from secondary bacterial, viral and protozoan infections (33, 142, 250) and confound vaccination efficacy, e.g. against malaria (215, 240).

2.1.1 Immune responses to intestinal nematode infections

A typical finding for GI nematode infections is a strong T helper cell type 2 (Th2) response in the mammalian host. This type of humoral immune response is generally seen as a combination of both innate and adaptive components and typically characterized by the production of the cytokines interleukin-4 (IL-4), IL-5, IL-9, IL-13, IL-21 and IL-25 by $CD4^+$ T cells (6, 40, 41, 235). While Th1 responses are critical for protection against protozoan, bacterial and viral infections, it is clear that Th2 cytokines mediate protection against intestinal nematodes (67, 237).

The key Th2 cytokines IL-4 and IL-13 lead to B cell isotype switching and IgE production. Th2 cells are activated and expanded in presence of IL-4, while the development of a Th1 response characterized by T cells producing IFN-γ is suppressed (158). Th2 cytokines also act on innate immune cells. They favour eosinophilia (IL-5, IL-4, IL-13) (66, 121) and increased numbers of basophils and mast cells (IL-9, IL-4, IL-13) (63, 64). Macrophages may be converted to an alternatively activated state by the action of IL-4 and IL-13, clearly differing from the nitric oxide producing classically activated macrophages (187). The Th2 response may be accom-

panied by an increase of IL-10 secreting T cells, B cells and macrophages. IL-10 was initially described as a Th2-related cytokine with anti-inflammatory function, deactivating most hematopoietic cells (155). More recently it has been demonstrated that also Th1 cells produce IL-10 (166, 104) and that it downregulates both Th1 and Th2 mediated inflammation (75, 197, 209). IL-10 provokes IgG4 isotype class switching while inhibiting IgE production by B cells (105). In addition, IL-10 is described as anti-apoptotic factor for B cells sustaining the humoral immune response (101).

Besides the effects of Th2 cytokines on adaptive and innate immune cells, they also act on non-hematopoietic cells. During GI nematode infections, IL-4 and IL-13 provoke goblet cell hyperplasia, leading to increased mucus production (67). Both cytokines also act on epithelial and smooth muscle cells, leading to increased tissue permeability, fluid influx to the intestine, muscle hypercontractility, as well as an increased epithelial cell turnover, thereby leading to parasite stress and reduced fecundity of adult worms (6, 40, 66, 67, 121, 202, 259). This so-called “weep and sweep” reaction may favour expulsion of live worms due to unfavourable conditions (203).

It is still under debate whether the Th2 response to worm infections reflects the appropriate form to combat such parasites or whether helminths have evolved to stimulate Th2 responses for their own advantage (139). The outcome of human infections with fluke worms (*Schistosoma spp.*) or filarial nematodes (*Brugia*, *Onchocerca)* is highly dependent on the T helper response, with certain life stages provoking a Th1 response, while others favour a switch to a predominant Th2 response in most patients and animal models. This is expressed in marked differences with respect to immunopathology and parasite load. Disparately, GI nematodes provoke highly polarized and rather stereotypical Th2 responses associated with protection (130). This polarization is induced independently of the mucosal milieu of the infection site, as application of intestinal parasites directly to the skin also induces a Th2 response (130). Interfering with this response may lead to increased intestinal pathology and block protective immunity. Hence, Th2 responses are beneficial for the host. On the other hand, the responses are carefully controlled to reduce pathology, thereby eventually leading to long-time persistence of the pathogen with ongoing transmission (138).

2.2 *H. polygyrus* as a mouse model for GI nematode infections.

The most widely used mouse models for GI nematode infections are *Trichuris muris*, *Heligmosmoides polygyrus*, and *Nippostronglyus brasiliensis*. The adults produce high numbers of eggs shed by the host for subsequent transmission. As an example, mice infected with approximately 150 adulti of *H. polygyrus* show counts of up to 80.000 viable eggs per gram of feces. The nematodes occupy different niches within the intestine: the lumen (*N. brasiliensis*), the epithelium (*T. muris*) or the mucosal surface (*H. polygyrus*). Different inbred mouse strains may vary in their immune response to infection and enteropathy depends on direct damage of host tissue due to feeding, penetration, migration of larval forms, or secondary damage by bacterial infections (73, 121).

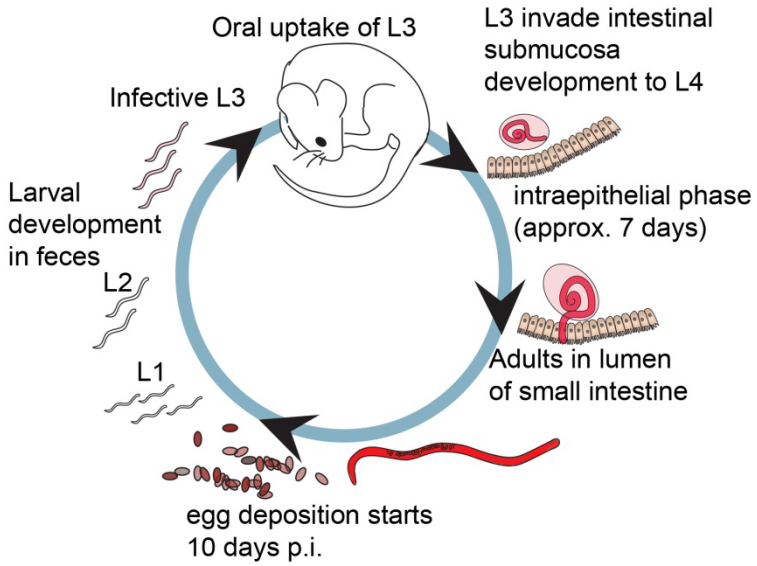

Fig 1. Life cycle of *H. polygyrus.* *H. polygyrus*-infection is strictly enteric and follows oral uptake of free-living infective larvae (L3). After ingestion, the larvae invade the gastric and duodenal submucosa within hours. Here the development to L4 takes place (histotrophic phase). The L4 reenters the gut lumen around day 8 post infection, rapidly develops to the adult form, which starts with egg deposition by 9 days post infection (154). The L3 develops in feces via L1 and L2 within 5-7 days. Primary infections in mice are chronic, while secondary infections after drug-cure are expelled within 14 days (74).

The trichostrongyloid *H. polygyrus* serves as a model organism for human hookworm infections. While *Ancylostoma* and *Necator* invade their host percutaneously and migrate through

the host via bloodstream, lungs and trachea until becoming swallowed, *H. polygyrus* has a strictly enteric life cycle (Fig. 1). Mice are infected by oral uptake of infective L3, which penetrate the gastric and small intestinal submucosa for further development to the L4. After this short histotrophic phase, the mature adults are found within the anterior part of the small intestine (Fig. 1). The infection provokes a highly polarized Th2 cell response with marked increase of IL-4, IL-5 and IL-13, leading to eosinophilia, and increased IgE and IgG1 levels in sera (221, 236, 243). As infections with *H. polygyrus* are chronic this parasite is a valuable model to study immunomodulation by long-lasting infections. Infections take a rather mild course in comparison to those with other GI nematodes, such as *Trichinella spiralis* leading to heavy intestinal inflammation (R. Grencis, personal communication). While human hookworms feed from host tissue and blood, leading to permanent blood losses and tissue damage, adult *H. polygyrus* feeds mainly on mucosal tissue. (10). Infection with high numbers of adult worms (~ 200) lead to little morbidity and low weight loss under laboratory conditions (33).

Secondary infections are even less severe than primary infections with accelerated worm expulsion, lower fecundity and smaller adult worms (45). However, protection is generally not complete (261). Abrogation of primary infections during the intraepithelial larval phase enhances resistance to secondary infections by a strengthened immune response (59). These features show that *H. polygyrus* is a well-suited model for the investigation of a protective memory response against GI nematodes.

2.3 Immune response to *H. polygyrus*

Infection with *H. polygyrus*, although completely enteric, induces a systemic Th2 response (153, 201, 221, 243). This impairs the development of Th1 responses against unrelated antigens (14) or pathogens (33, 34, 107) and confound vaccination efficacy (214, 215).

2.3.1 The impact of the hosts genetic background

Primary infections with *H. polygyrus* are chronic in most mouse strains, however the genotype of the mouse correlates with resistance. The Th2 response against *H. polygyrus* as seen

by increased IgG1 and IgE serum titers and eosinophilia is a general feature in all investigated mouse strains (18, 179). However, inbred strains vary with respect to survival after infection with a lethal dose of parasites (170), the ability to control development and reproduction of the parasite (50) and the protection against reinfection (26, 179). Genes linked to the H-2 complex have been shown to correlate with variation in resistance to *H. polygyrus*, but other factors are not excluded (213). These differences lead to the distinction between slow, intermediate and fast responder mouse strains. The BALB/c mouse strain displays an intermediate susceptibility with high worm loads during the first weeks after primary infection and few worms persisting for longer periods of time (personal observation). The C57BL/6 and CBA strains are examples for slow responder strains with high susceptibility to primary infections, long persistence of high adult worm burdens and high fecal egg counts. Secondary infections are less severe, but still adults may be maintained for extended time periods. Inbred SJL, SWR, DBA/I and outbred RH mice are fast responder strains with high resistance towards secondary infections and low fertility of worms in both primary and secondary infection (16, 17, 24).

Taken together, these findings argue for qualitative differences of the Th2 response in different strains correlating with resistance. However, the nature and direct impact of these differences on protection are not fully elucidated.

2.3.2 Adaptive and innate immune mechanisms.

It is clear that $CD4^+$ cells and the Th2 cytokines IL-4 and IL-13 are critical for protection against GI nematodes, while Th1 cytokines may promote survival of the parasite (57, 65, 67). Studies on immune responses against *H. polygyrus* showed that resistance against secondary infections is strictly dependent on $CD4^+$ Th2 cells producing IL-4 and IL-13 (7, 238, 239 and Fig. 2). Although the resistance to secondary infection with *H. polygyrus* correlates with total IgE and IgG1 levels and with the strength of eosinophilia in mice (17, 261), there is no striking evidence for a role of parasite-specific IgE in mediating resistance against this nematode (6). Still, B cells take part in IL-4 production, although this effector function is subordinate to that of other cell types, such as T cells, eosinophils and mast cells (87,153). A recent study with B cell deficient mice suggested that B cells sustain the Th2 response, as IL-4 production by T cells was markedly reduced in B cell deficient mice (128).

The mechanisms by which IL-4 and IL-13 induce protection against *H. polygyrus* were for long poorly defined. Recently, it has been shown that $CD4^+$ T cells producing both cytokines rapidly accumulate around larvae during a memory response, leading to protection with alternatively activated macrophages critically involved as innate effector cells.

These findings are less prominent during a primary infection, arguing for a delayed response permitting successful parasite development (6). However, even in secondary infections, not all worms are removed, arguing for a second immunomodulatory defence strategy of the parasite. One advantage of *H. polygyrus* might be the short tissue dwelling phase during development. This could favour a delayed or insufficient Th2 response, while parasites with larval stages migrating through tissue (*N. brasiliensis*, *T. spiralis*) or adults in permanent contact to the epithelial compartment (*T. muris*) may stimulate responses of a higher magnitude, leading to faster expulsion.

Neutrophils and eosinophils are the first effector cells appearing in the granuloma around tissue-embedded larvae after primary and secondary infection (see Tab. 1) and intraepithelial mast cells rapidly produce IL-4 and IL-13 after infection with *H. polygyrus* (76, 153). Although correlations between resistance and strength of eosinophilia and mastocytosis exist (17, 28, 261), most studies imply a minor role for these cells in combating the parasite (6, 114). The role of neutrophils is poorly examined. As these cells are among the first recruited to the site of infection (see Table 1), they are thought to take part in mediating parasite damage, probably in combination with eosinophils and macrophages. Recently, it has been shown that cells of a neutrophil-like phenotype accumulate in the granulomas around tissue invasive larvae and that these cells take part in IL-4 and IL-13 production at the site of infection (7). Dendritic cells (DC) have been shown to be activated and express IL-10 in response to *H. polygyrus* infection. Hence, DC probably take part in suppressing the immune response against the parasite, but they are also impaired in their responses against enteric bacterial infections (33, 34).

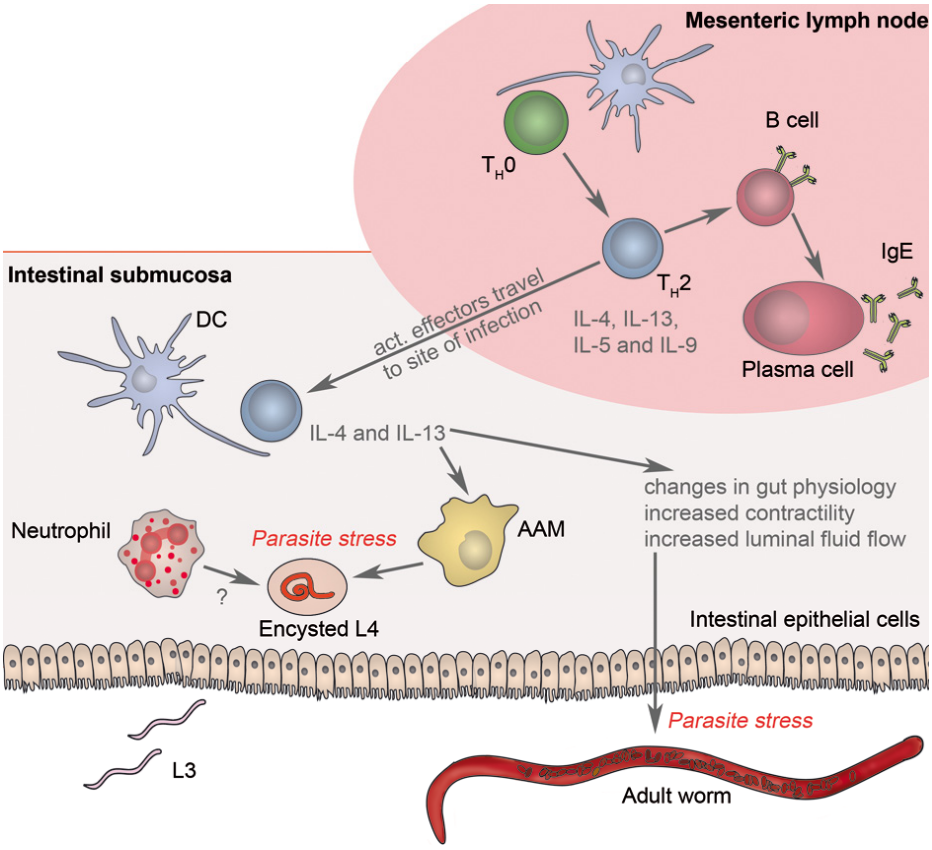

Fig. 2 Immune response against *H. polygyrus*. Infective larvae (L3) are ingested by the host, enter the gastric and small intestinal submucosa, develop to L4 and adults are found in the lumen of the SI at day 8 pi. Parasite antigen is presented to $CD4^+$ T cells in mesenteric lymph nodes and other gut-associated lymphoid tissues, driving the development of Th2 cells. These migrate to the site of infection. By secreting Th2 cytokines, T cells provoke B cell immunoglobulin isotype switching to IgE and IgG1, alternate activation of macrophages (AAM) and changes in gut physiology. An immune cell infiltrate develops around the encysted larvae especially during secondary (2°) infections, comprised by neutrophils, AAM, $CD4^+$ T and dendritic cells. This leads to increased parasite stress during 2° infections. Changes in the gut physiology probably lead to unfavourable conditions for adult worms, eventually resulting in worm expulsion. (Adapted from Ref. 6)

Cell type present in granuloma		1° infection	2° infection
CAM		-	-
AAM		-	+++
Neutrophil		++	++
DC		+	++
Eosinophil		++	++
Th1 cell		-	-
Th2 cell		-	++

Tab.1 Cellular composition of *H. polygyrus*-induced granulomas (adapted from Ref. 6)

Macrophages are conventionally associated with Th1 responses against bacterial and protozoan infections. Recently it has become clear that macrophages also play a central role in Th2 immune reactions against helminths, such as filaria (82, 227) and schistosomes (91). These cells show an alternatively activated phenotype (AAM) and are involved in tissue repair, but also in controlling excessive inflammatory responses induced by trapped schistosome eggs and tissue dwelling parasites. Infection with *H. polygyrus* was also shown to induce AAM expressing the IL-4R, CD206 and arginase-1. These cells are critically involved in rapid expulsion of secondary infections, with the larval stage as target. This is the first definitive finding that innate immune cells with a specific activation status are involved in mediating protection against *H. polygyrus* (7). Others showed that *H. polygyrus*-induced AAM impair immunity to bacterial pathogens and may thereby lead to exacerbated intestinal injury by bacterial infections (250).

2.3.3 Physiological changes induced by Th2 responses

IL-4 and IL-13 are shown to act on epithelial, goblet and smooth muscle cells in the small intestine, creating an inhospitable environment for GI nematodes (121, 203). While goblet cell hyperplasia is dependent on IL-13 (147), IL-4 is the major player in *H. polygyrus*-induced changes of epithelial cell function (202). Both cytokines induce changes in smooth muscle contractility of the intestine. This has been shown for secondary infections with *H. polygyrus* and can be mimicked by application of exogenous IL-4 or IL-13 and blocked by treatment

with anti-IL-4 receptor antibodies (202, 259). The effect of IL-13 on smooth muscle cells is almost entirely dependent on signal transducer of activation and transcription 6 (Stat6) and is manifested as an increased contractility in response to the neurotransmitter acetylcholine (259). IL-4 probably increases smooth muscle hypercontractility by an effect dependent on mast cells producing leukotriene D_4, which increases the sensitivity of nerves for stimulation (259). Increased motility of the small intestine might lead to limited access to the food resource and thereby lead to parasite stress.

IL-4 also influences the influx and absorption of fluid in the intestine. Secondary infections with *H. polygyrus* induce a higher permeability of the intestine, leading to increased fluid influx. This is accompanied by reduced fluid absorption in response to glucose. These effects are again dependent on $CD4^+$ T cells. While the contribution of mast cells to this phenomenon is less clear for *H. polygyrus* (17), increased epithelial paracellular permeability is strictly dependent on mast cell function in infections with other intestinal nematodes (144).

2.4 Regulatory T cells (Tregs)

Regulatory T cells represent a subset of $CD4^+$ T cells that are critically involved in balancing the reactivity of the immune system and preventing autoimmunity (141, 191, 192). Treg cells suppress effector T cells by means of activation, cytokine production, cytolytic function and expansion (204, 242). Naturally occurring regulatory T cells (natural Tregs) are thymus-derived $CD4^+$ cells expressing a T cell receptor repertoire of a broad variety and activation by TCR- signalling is critical for their suppressive function (204, 230). Until recently, such Tregs were considered to mainly recognize self-antigens (96), thereby controlling undesired activation of other self-reactive T cells. Now it is becoming clear that rather few Tregs express T cell receptors (TCRs) specific for self-antigens (172).

Tregs are phenotypically characterized by the expression of an array of surface markers, such as CD25 (the α-chain of the IL-2 receptor), CTLA-4 (CD152; cytotoxic T-lymphocyte antigen-4) and GITR (glucocorticoid-induced tumor necrosis factor receptor family-related protein). CD45RB, CD62-L, neuropilin (NRP)1 and the lymphocyte activation gene (LAG)-3 show enriched expression in Treg population, while the downregulation of the IL-7 receptor (CD127) distinguishes Tregs from activated T cells. In addition, the integrin α_E(CD103)β_7 is a marker for

a subset of highly potent effector/memory-like Tregs, and $CD4^+CD103^+$ cells were found to be the most potent suppressors of inflammatory processes in disease models such as colitis and arthritis (97, 98, 124, 205). None of the given markers is exclusive for Tregs. CD25 is also expressed in recently activated effector cells, while Tregs constitutively express this marker in high densities. Tregs depend on IL-2 provided by conventional T cells, as they are unable to produce IL-2 (13, 46). This is seen in animals deficient in IL-2 or with a defective IL-2-receptor, developing uncontrolled auto-inflammatory T cell responses and virtually no Tregs, clearly pointing out that IL-2 is needed for Treg maintenance, activation and expansion in the periphery (4, 140).

To date, the most reliable marker for naturally occurring Tregs is the *forkhead* family transcription factor Foxp3 (forkhead box protein P3) (69). This marker is expressed in mouse as well as human Tregs, although activated human conventional T effector cells may upregulate Foxp3 under certain conditions (244). Ectopic expression of Foxp3 in conventional T cells renders them functionally inert, with a decreased proliferative response to ligation of their TCR and abrogated IL-2 expression, while enabling them to suppress the activation of other T cells (69, 95). There is evidence that Foxp3 acts as a transcriptional repressor of cytokines. Binding sites for Foxp3 are found overlapping with or directly adjacent to the binding sites for NFAT and AP-1, and binding of Foxp3 may regulate the expression of the cytokines (199). A direct interaction of Foxp3 with NFAT and NF-κB is also described (27).

2.4.1 Subsets of Tregs and mechanisms of suppression

The suppressive mechanisms of Tregs are not fully elucidated. The general notion is that the mechanisms of suppression differ with respect to, first, the conditions analyzed (*in vitro* versus *in vivo*) and, second, depend on the subset of regulatory T cells.

Natural Tregs ($CD4^+CD25^+Foxp3^+$) are found under homeostatic conditions and need cell contact to suppress the activation of conventional T cells *in vitro* (204, 242). They do not proliferate *in vitro* unless stimulated via their TCR in presence of exogenous IL-2. They suppress the activation and expansion of $CD4^+$ and $CD8^+$ T cells in absence of antigen presenting cells (APC) and independently of soluble mediators like IL-10 and TGF-β (176, 223). Once such Tregs were activated via their TCR, the suppression is completely Ag un-specific (231). The

inhibition of IL-2 production by responder cells is seen as the hallmark of their action on conventional T cells (230).

So-called adaptive Tregs are induced in the periphery from non-regulatory, $Foxp3^-$ precursors. Induced Tregs appear in the context of infections and include converted $Foxp3^+$ T cells that are indistinguishable from natural Tregs. Tr1 and Th3 cells are seen as (mostly $Foxp3^-$) induced Tregs exerting their suppressive function *in vitro* and *in vivo* by the secretion of the anti-inflammatory cytokines IL-10 and TGF-β (21, 51, 194). IL-10 producing Tr1 cells may be induced in the presence of deactivated or immature antigen presenting cells (151) or the prolonged exposure to antigen or IL-10 itself (167). IL-10 and TGF-β-producing Tregs are essential for suppression of intestinal inflammation (9) and T cells with a defect in TGF-β-receptor signalling escape suppression by Tregs *in vivo* (58). A second feature of TGF-β is the contribution to the generation and proliferation of natural Tregs. Thereby this cytokine generally favours immunosuppression (41, 72).

Surface molecules engaged in suppression were extensively analyzed. One candidate is CTLA-4, a homologue to CD28, which is constitutively expressed on Treg cells, while conventional T cells express it after activation (224). CTLA-4 is a suppressive costimulatory molecule that competes with CD28 ligation by CD80 and CD86 (B7 molecules) expressed on activated $CD4^+$ cells, thereby deactivating effector T cells. This mechanism is referred to as “outside-in” signalling of B7 molecules and may explain the suppression seen in APC-free cultures (175, 229). CTLA-4 may also bind to B7 molecules on APC, leading to expression of indoleamine 2,3-dioxygenase (IDO) and reduced free tryptophane levels, which renders it more difficult to activate T effector cells (148). This effect is not essential *in vitro*, as suppression is also seen in APC-free cultures. Furthermore, CTLA-4 might also be involved in mutual regulation of T cell activation in the absence of Tregs (242). The engagement of CTLA-4 in mediating suppression *in vivo* could be shown in a graft-versus-host disease model, where Tregs were only able to suppress inflammation when the effectors expressed the B7 molecules. B7 knock-out effector cells could not be controlled (175). Evidence for the role of this molecule in immune suppression also comes from CTLA-4 knock-out mice with severe lymphoproliferative disorders (248). Finally, an enhanced engagement of CTLA-4 on T cells by DC may induce adaptive Treg cells (127).

A role for surface bound TGF-β on Tregs in contact-dependent immunosuppression is also described (159), however others have shown that the addition of antibodies against TGF-β or soluble TGF-β-receptor II does not interfere with suppression by Tregs *in vitro* and that T cells with a dominant negative TGF-β-receptor are efficiently suppressed by Tregs (176).

An additional *in vivo* mechanism for suppression by Tregs is the inhibition of stable contacts between T effector cells and dendritic cells in lymph nodes, probably hampering the activation of T effector cells at the initial phase of an immune response (222). Consequently, Tregs interfere with T cell activation *in vivo* via the modulation of APC functions.

There is clear evidence that suppression by Tregs is reversible, excluding the induction of increased apoptosis as a major factor for suppression of $CD4^+$ effector cells. T effector cells suppressed by contact with Tregs restart to produce IL-2 after Treg removal. Similarly, Th1 effector cells may still produce IFN-γ in Treg cocultures after stimulation with PMA/Ionomycin (a form of stimulation circumventing TCR engagement on both T cell subsets) (113).

2.5 Tregs and infection

Common effector mechanisms against pathogens include the production of pro-inflammatory cytokines and chemokines, recruitment of inflammatory cells to the site of infection and activation of T helper, cytotoxic and natural killer T cells. These responses eliminate or slow down the spreading of pathogens, but need to be tightly controlled in order to avoid excessive damage to host tissue. Damage may also arise from immune responses against self-antigens under inflammatory conditions, eventually leading to the development of autoimmune reactions (151). Therefore the immune system developed several circuits to control undesired and exaggerated immune reactions, including the production of anti-inflammatory cytokines by innate immune cells.

Highly effective immune evasion mechanisms are key elements of the long-lasting persistence of parasitic worms. They have evolved mechanisms to manipulate the host's immune response to ensure their prolonged survival and transmission (138, 139) and both parasite-specific and non-specific immune suppression by helminths are well documented (86, 178,

252). One form of immune evasion is to exploit the host's mechanisms for controlling collateral damage to own tissue by addressing regulatory networks of the immune response. Hence, the generation or activation of Tregs may be favoured by helminths (138, 228).

Tregs have been shown to play an essential role in the outcome of viral (102), bacterial (117, 118), protozoan (94) as well as helminth infections (51, 194). In some cases, prolonged parasite survival can be linked to Treg suppression of host protective responses (23, 118, 162, 241), while in others Tregs downmodulate potentially pathological immune responses (92, 181, 182) (see also appendix). Mice resistant to infection with *Leishmania major* serve as an example for the first case, as they are unable to eradicate the pathogen due to the accumulation of natural Tregs at the site of infection (23). The role of Tregs in controlling pathology is seen in mice infected with schistosomes (fluke worms), where removal of Tregs causes liver damage due to strong inflammatory responses to the parasite eggs (92). In humans infected with *Plasmodium falciparum*, removal of Tregs enhances parasite-specific effector responses (246), while Tregs control fatal malaria in animal models (162). The *in vivo* depletion of regulatory T cells leads to parasite clearance in experimental filarial infections (228) and parasite specific clones of Tregs suppressing effector responses have been isolated from filariosis patients (51, 194). While it is clear that Treg cells induced in the periphery may show specificity for microbial antigens (145, 151, 51, 194), there is increasing evidence, that also natural Tregs recognize pathogens (25, 92, 146, 216,). The discrepancy with the general view that natural Tregs recognize self-antigens is obvious. However, self-specific Tregs are probably required to control undesired inflammatory responses spilling over to auto-antigens due to tissue injury and increased presentation of host antigens during the acute phase of an infection. Still, regulatory cells indistinguishable from natural Tregs (with respect to expression of CD25 and Foxp3) and specificity for pathogens are predominantly detected in chronic infections. This might argue for long-lasting infections as a prerequisite for the development of natural Tregs with specificity for pathogens (25). Furthermore, recent studies revealed that the TCR repertoire of thymus-derived natural Tregs is not restricted to self-antigens (172).

In this work, *H. polygyrus* was used to investigate the role of different $CD4^+$ T cell subsets during the chronic phase of a primary nematode infection. Motivated by the lack of information on the activity of $CD4^+$ T cell subsets during chronicity (when T cell reactivity is sup-

pressed) and on the T cell subsets that contribute to host protective or parasite beneficial immune responses, $CD4^+$ effector (Teff) cells and regulatory T cells were analyzed in adoptive transfer studies with regard to worm expulsion and the role of Tregs to modulate T effector cell function. Transfer of effector cells led to protective immune responses in which antigen-specific $CD4^+$ T cells produced predominantly IL-4 and IL-13. In contrast, Tregs from the chronic phase of infection showed no effect on adult worm burden after adoptive transfer, although producing IL-10 and being highly potent suppressors *in vitro*.

2.6 The filarial cystatin Av17

The cystatin of *Acanthocheilonema viteae* (Av17) is described as an immunmodulatory protein secreted by this tissue-dwelling filarial parasite (88, 89, 195). It belongs to the cystatin superfamily, which is comprised of evolutionary conserved, tight-binding and reversible inhibitors of cystein proteases (161). Cysteinproteases exist in virtually all organisms and are involved in a broad spectrum of biological processes, such as protein metabolism, processing of antigens as well as inflammation, and they need to be regulated by cystatins. There are three major families of cysteine protease inhibitors: stefins, kininogens and cystatins. Filarial cystatin shows high homology to human cystatin C, which is constitutively expressed and may be secreted by human mononuclear phagocytes (247). Filarial cystatin is secreted by all stages of the parasite and could be shown to inhibit the immunologically relevant human cathepsins L and S (196), thereby interfering with the processing and presentation of antigen by macrophages. Furthermore, other nematode cystatins have also been shown to inhibit the activity of cysteine proteases (89). Hence, these parasite-derived protease inhibitors are seen as immunomodulators of the host immune response. They interfere with antigen-specific T cell responses by suppressing antigen processing and loading of MHC class II complexes with peptides by APC. Thus, parasite cystatins are seen as regulatory proteins that interfere with downstream immune reactions by modulating APC functions, especially that of macrophages (89).

In this study, the immunomodulatory cystatin Av17 was tested for effects in a mouse model of colitis.

2.7 The DSS Colitis Model

Chemically induced intestinal inflammation models are very common for the investigation of pathogenesis of Crohn disease (CD) or ulcerative colitis (UC), the primary constituents of human inflammatory bowel disease (IBD). Both forms of IBD are constituted by a complex interaction of environmental, genetic, and immunoregulatory factors, while a higher incidence of IBD is seen in industrialized countries. Epidemiological as well as human and animal experimental data support the postulate that underexposure to helminths predisposes to IBD (249). Regardless of the underlying genetic predisposition, a dysfunctional mucosal immune response to commensal bacteria is implicated in the pathogenesis of IBD (84, 177). Dextran sodium sulphate (DSS) is among the most commonly used chemicals to induce colitis in animal models. The contribution of the intestinal microflora to the development of colitis in this model was shown by the use of germ-free animals, displaying only minor intestinal reactions to the chemical (232). For human IBD, possible triggers for disease onset include a chronic inflammatory response against infection with pathogens or viruses or a defective mucosal barrier. The characteristic inflammatory response begins with an infiltration of neutrophils and macrophages, which then release chemokines and cytokines. These in turn exacerbate the dysfunctional immune response and activate either Th1 or Th2 cells in the gut mucosa, associated with CD and, less conclusively, with UC (84). Although clearly limited, chemically induced colitis models share immunological and histopathological aspects with human IBD (253). DSS seems to be directly toxic to colonic epithelial cells, thereby destroying mucin content and disrupting the barrier function of the epithelium. This leads to inflammation due to increased exposure to luminal contents and altered macrophage functions (112, 160, 168). Feeding mice with DSS-polymers in drinking water induces acute and chronic inflammation characterized by body weight loss, bloody diarrhea, ulcerations and mononuclear cell infiltrations (mainly granulocytes), resembling to human ulcerative colitis (168). The treatment provokes regenerative changes of the epithelium. T and B cells are not essential during the acute phase of disease development, as also SCID or $RAG^{-/-}$ immunodeficient mice develop colitis in response to DSS feeding (49). Therefore, this model is particularly useful to study the contribution of innate immune mechanisms to colitic inflammation. However, when applying DSS for longer periods (the chronic form of the model), also T cells are involved in inflammation

(B. Wittig, personal communication) and it has been shown, that even short time application of DSS provokes an elevated production of IL-4 and IFN-γ by T cells (48). It is clear from studies with IFN-$\gamma^{-/-}$ mice, that this cytokine is a central player in mediating inflammation in this model (100). Furthermore, intestinal macrophages are critically involved in controlling inflammation in response to DSS, as their depletion increases severity of the disease, probably due to uncontrolled neutrophil infiltration (180). Taken together, besides polymorph nuclear cells and macrophages, also T helper cells are involved in chronic inflammation provoked by DSS treatment.

During the last years, rising evidence was found for the beneficial effect of worm infections or the application of helminth eggs in a variety of colitis models (55, 56, 70, 107, 150, 156, 201, 208). First clinical trials also showed such effects on human IBD patients (217, 218). Instead of working with worm eggs or infection, the focus of this work was the application of a parasite molecule with defined immunmodulatory properties. The hypothesis was that the filarial cystatin Av17, a molecule with anti-inflammatory features, might interfere with the development of colitis in the aforementioned mouse model, especially as this model of intestinal inflammation is described to involve the action of macrophages, which have been characterized as target cells of Av17 in previous studies

3 Results

3.1 Analysis of lymphocytes in MLN of mice chronically infected with *H. polygyrus*.

To gather information about changes occurring in the mesenteric lymph nodes (MLN) after infection with *H. polygyrus*, the lymphocyte numbers and composition were surveyed during primary infection. Cell numbers of MLN showed a significant increase during infection ($p<0.0159$), while flow cytometric analysis revealed a decline in $CD4^+$ T cell frequencies (Fig 1). Comparing naïve to chronically infected animals (28 dpi) revealed a 5.2-fold increase ($p<0.05$) of total MLNC in infected compared to naïve animals (Table 1). A 4.7-fold ($p<0.05$) increase of absolute $CD4^+$ and a 4.3-fold ($p<0.05$) increase of absolute $CD8^+$ T cell numbers were detected by flow cytometry. The strongest increase in total numbers was found for B cells (8.3-fold, $p<0.05$) (Table 1). Interestingly, the number of $CD25^+CD103^+$ T cells increased 7.8-fold ($p<0.05$) within the $CD4^+$ T cell compartment, surpassing the outgrowth of $CD25^+CD103^-$ T cells (5.1-fold, $p<0.05$) and $CD25^-CD103^-$ T_{eff} cells (4.5-fold, $p<0.05$) (Table 1). As CD103 expressing $CD4^+$ T cells have been described as potent regulatory T cells (124), Tregs were analyzed in detail during primary *H. polygyrus* infection.

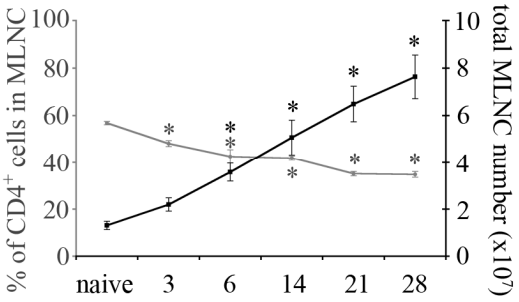

Fig. 1. MLNC numbers and $CD4^+$ T cell frequency during infection with *H. polygyrus.* Mean and SEM of 4-5 animals per time point is shown. Asterisks indicate statistically significant changes compared to naïve controls. Data are representative for three independent experiments.

TABLE 1. Total MLNC numbers and lymphocyte composition of MLN from naïve and *H. polygyrus* infected animals.

		naive	28 dpi	fold increase
total number of cells		$1.72 \times 10^7 \pm 0.29$	$8.96 \times 10^7 \pm 1.1$ *	5.2
$CD4^+$		[b] $9.32 \times 10^6 \pm 1.40$	[b] $43.54 \times 10^6 \pm 5.17$ *	4.7
	[a] $CD25^-CD103^-$	$8.28 \times 10^6 \pm 1.21$	$36.93 \times 10^6 \pm 4.35$ *	4.5
	$CD25^+CD103^-$	$0.79 \times 10^6 \pm 0.12$	$4.06 \times 10^6 \pm 0.29$ *	5.1
	$CD25^+CD103^+$	$0.21 \times 10^6 \pm 0.06$	$1.64 \times 10^6 \pm 0.28$ *	**7.8**
$CD8^+$		$2.89 \times 10^6 \pm 1.01$	$12.71 \times 10^6 \pm 3.19$ *	4.3
B cells ($CD19^+$)		$4.67 \times 10^6 \pm 2.12$	$38.99 \times 10^6 \pm 15.54$ *	**8.3**

[a] $CD4^+$ T cell subsets.

[b] Mean $\pm$ SEM of four (naïve) and five (28 dpi) animals. Similar data were obtained in four experiments.

* Statistical significance for comparison of naïve to infected animals as determined by Mann-Whitney test ($p < 0.05$).

3.2 Analysis of Tregs during the course of infection.

To investigate changes within the regulatory T cell compartment in response to *H. polygyrus* infection, Tregs were analyzed by the combined detection of the surface markers CD4 and CD25 and Foxp3. To discriminate between naïve-like natural and effector/memory-like Tregs, the integrin α_E(CD103)β_7 was detected (Fig. 2). Using this marker combination, the frequencies as well as phenotypic changes of Tregs in mesenteric lymph nodes and spleen of *H. polygyrus* infected mice were characterized at different time points after infection. Within the MLN draining the site of infection, a significant increase ($p<0.03$) in $CD25^+CD103^+$ T cell numbers was detected as early as day 6 pi in comparison to naïve animals (Fig. 3A). Within the spleen, a significant increase in Treg numbers was determined from day 12 pi onwards ($p<0.03$) (Fig. 3B). The higher frequency of T cells expressing CD103 was stable in both lymphoid compartments until the chronic phase of infection (Fig. 3A, B) and returned to the level of naïve mice when adult worms were expelled after 8 to 12 weeks (data not shown). $CD25^+CD103^-$ Tregs showed no such increase (data not shown).

Flow cytometric analysis showed that Foxp3 expression levels were >90% in $CD4^+CD25^+CD103^-$ as well as $CD4^+CD25^+CD103^+$ cells at most time points analyzed, clearly pointing out the regulatory phenotype of both subsets (Fig 4A). Carefully comparing the percentages of $Foxp3^+$ cells within the $CD4^+CD25^+CD103^-$ subset revealed a significant decrease in Treg numbers at day 6 pi (naïve 95.75±0.05%, 6dpi 86.72±1.11; $p<0.03$), arguing for an increased proportion of recently activated effector cells present in MLNC at this time point (Fig. 4A, upper panel). The $CD4^+CD25^+CD103^+$ subset displayed Foxp3 expression levels of >96% at all time points analyzed (Fig. 4A, lower panel), while few (<1.8%) $CD4^+CD25^-CD103^-$ cells expressed Foxp3 (not shown).

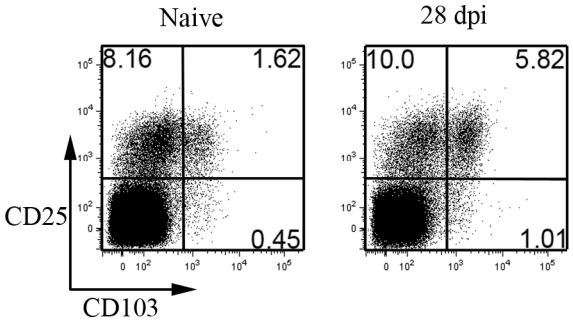

Fig. 2 Detection of Tregs by flow cytometry. Tregs were detected in lymphatic organs by surface expression of CD4, CD25 and CD103. Shown are exemplary plots for $CD4^+$ T cells from MLN. Naïve-like Tregs (upper left quadrant) separate from effector/memory-like Tregs (upper right quadrant) according to CD103 expression. A comparison between naïve and chronically infected animals is shown. Data are exemplary for 5 animals and representative for 5 experiments.

Comparing percentages of the total $CD4^+Foxp3^+$ population between naïve and infected animals (3, 6, 21 and 28 dpi) revealed no significant differences (Fig. 4B and not shown). Thereby the increase in total Treg numbers during infection reflected the increase of $CD4^+$ cell numbers. When analyzing the ratio of $CD103^+$ and $CD103^-$ Tregs after gating on all $Foxp3^+$ cells, a significantly decreasing proportion of $CD25^+CD103^-$ cells was detected in infected animals at the chronic stage ($p<0.0159$), counterbalancing the significant increase in $CD25^+CD103^+$ cells ($p<0.0159$) (Fig. 4C). Hence, significant changes concerning the frequency of effector/memory-like Tregs were detected after infection with *H. polygyrus.* This finding argues for a conversion of naïve-like natural Tregs into effector/memory like Tregs probably directly related to the infection.

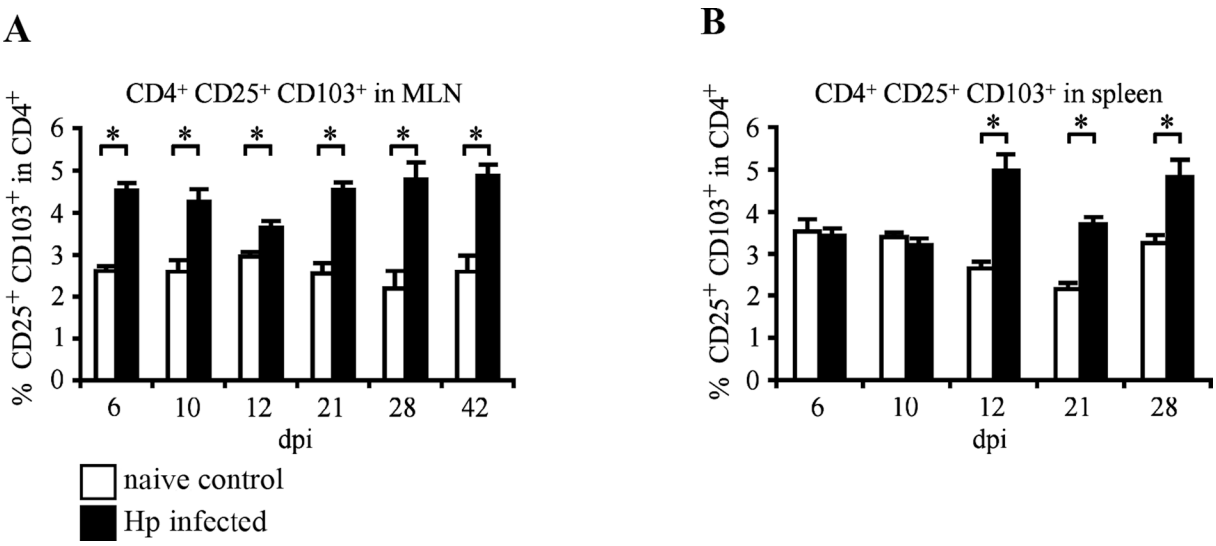

Fig. 3 Kinetic of effector/memory-like Tregs detected by flow cytometry. Frequencies of $CD25^+CD103^+$ cells in $CD4^+$ cells of MLN (A) and spleen (B). Percentages of Treg numbers within naïve controls (white bars) and infected animals at different time points after infection (black bars) are shown. Data were derived from 4 to 5 animals per group and analysis of Treg numbers was performed at least twice for each time point. Mean + SEM is shown.

To test whether the changes in Treg numbers found in the lymphatic organs were also reflected at the site of inflammation, Foxp3 expressing cells were detected in sections of the proximal third of the small intestine (Fig. 5A; Foxp3 *in situ* staining was performed by C. Loddenkemper, Charité Berlin). In clear contrast to the findings in lymphatic organs, a transient maximal accumulation of $Foxp3^+$ cells was detected at day 6 pi ($p<0.016$), which gradually returned to the basal level seen in naïve animals at later time points of infection (Fig. 5B). By additional staining of CD3, the changes in T cell numbers and proportions of Tregs could be calculated. Similar to what was seen for Foxp3 expressing cells, a transient increase in T cells at the site of infection was detected at day 6 pi. As infection turned chronic (day 21 and 28 pi), the frequencies of T cells fell short of the levels detected in naïve control animals ($p<0.032$(Fig. 5 C). As the accumulation of Tregs surpassed the increase of $CD3^+$ T cell numbers in the acute phase, a maximal ratio of Tregs to total T cells ($p<0.016$) could be ascribed to this stage of infection (Fig. 5D). The kinetic of $Foxp3^+$ cells was mirrored by a maximal production of TGF-β1 (Fig. 5 E) and IL-10 (Fig. 5 F) in the small intestinal tissue at day 6 pi.

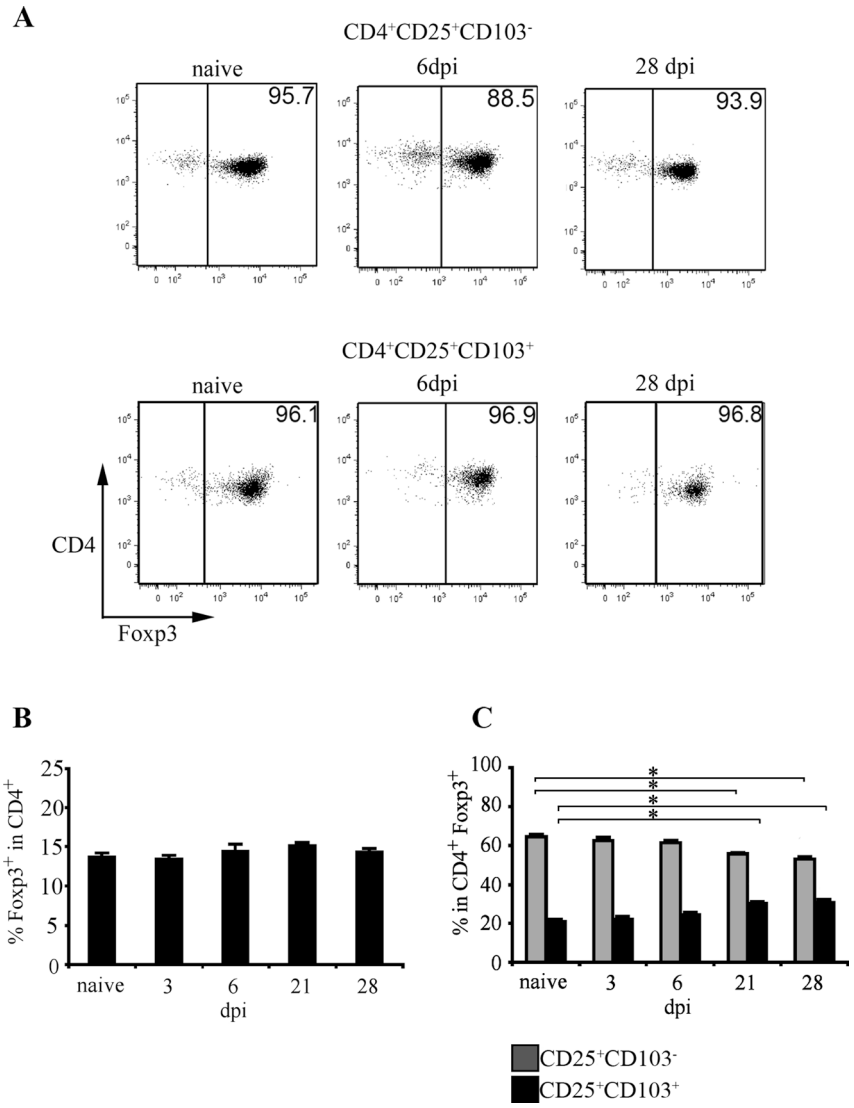

Fig. 4 Detection of Foxp3 expression and kinetic of effector/memory-like and naïve-like Tregs during infection. (A) The predominantly regulatory phenotype of $CD4^+CD25^+CD103^-$ (upper panel) and $CD4^+CD25^+CD103^+$ cells (lower panel) derived from MLN could be confirmed by intracellular staining of Foxp3. Four animals per group were analyzed in two independent experiments. Exemplary plots are shown. (B) Frequencies of total $Foxp3^+$ cells within $CD4^+$ lymphocytes from MLN. Mean + SEM of four animals per group is shown. Data are representative for two independent experiments. (C) Frequencies of naïve-like ($CD103^-$, grey bars) and effector/memory-like T_{reg} cells (black bars) in $CD4^+Foxp3^+$ cells. Mean + SEM of four animals per group is shown. Data are representative for two independent experiments.

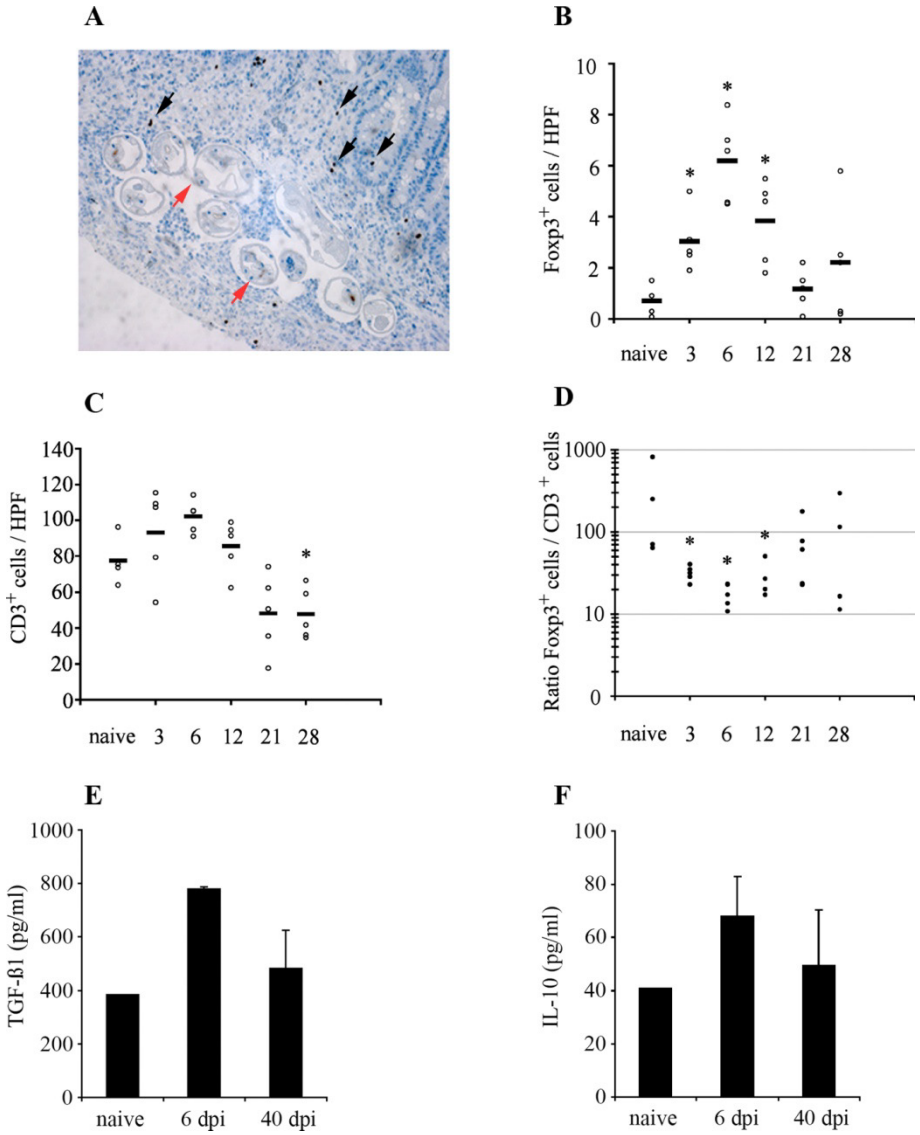

Fig. 5 Changes in T cell frequencies and cytokine production at the site of infection. (A) *In situ* staining of Foxp3$^+$ cells in a cross section of the proximal third of the small intestine (SI) 6 dpi. Black arrows: Tregs, red arrows: *H. polygyrus* L4 stage. (B,C) Kinetic of Foxp3 expressing cells (B) and CD3$^+$ T cells (C) at the site of infection. Cells in 10 high power fields (HPF, 40fold magnification) in sections of single animals were counted. 4 to 5 animals per time point were analyzed. Mean of animals (circles) and groups (lines) are shown. Asterisks indicate statistical differences compared to naïve controls. (D) Ratio of Foxp3 expressing Tregs to T cells in SI sections. Mean detected for single animals is shown. (E / F) TGF-β1 (E) and IL-10 (F) in homogenized SI samples as measured by ELISA. Samples were activated by acidification for TGF-β1 analysis. 3 to 6 animals per time point were analyzed. Mean + SD is shown.

Taken together, these data show that Tregs with an effector/memory-like phenotype increased strongly during infection with *H. polygyrus* in MLN and spleen, while Foxp3 expressing cells accumulated only transiently at the site of infection. This accumulation within the intestine surpassed the increase of total T cell numbers during the acute phase of infection and was accompanied by an increase in TGF-β1 and IL-10 levels at the site of infection.

In clear contrast to the findings in lymphatic organs, a transient maximal accumulation of $Foxp3^+$ cells was detected at day 6 pi ($p<0.016$), which gradually returned to the basal level seen in naïve animals at later time points of infection (Fig. 5B). By additional staining of CD3, the changes in T cell numbers and proportions of Tregs could be calculated. Similar to what was seen for Foxp3 expressing cells, a transient increase in T cells at the site of infection was detected at day 6 pi. As infection turned chronic (day 21 and 28 pi), the frequencies of T cells fell short of the levels detected in naïve control animals ($p<0.032$(Fig. 5 C). As the accumulation of Tregs surpassed the increase of $CD3^+$ T cell numbers in the acute phase, a maximal ratio of Tregs to total T cells ($p<0.016$) could be ascribed to this stage of infection (Fig. 5D). The kinetic of $Foxp3^+$ cells was mirrored by a maximal production of TGF-β1 (Fig. 5 E) and IL-10 (Fig. 5 F) in the small intestinal tissue at day 6 pi.

Taken together, these data show that Tregs with an effector/memory-like phenotype increased strongly during infection with *H. polygyrus* in MLN and spleen, while Foxp3 expressing cells accumulated only transiently at the site of infection. This accumulation within the intestine surpassed the increase of total T cell numbers during the acute phase of infection and was accompanied by an increase in TGF-β1 and IL-10 levels at the site of infection.

3.3 Suppressive capacity of Tregs from *H. polygyrus*-infected mice *in vitro*.

To further investigate the suppressive capacity of Tregs from worm-infected animals, sorted Treg subsets were analyzed *in vitro*. $CD4^+CD25^+CD103^+$ and $CD4^+CD25^+CD103^-$ Tregs were isolated from naïve and worm-infected mice in the acute (12 dpi) and chronic (28 dpi) phase of infection. The cells were added to Treg-depleted $CD4^+$ T cells from naïve mice, which were polyclonally stimulated to proliferate. $CD4^+CD25^+CD103^+$ Tregs from infected mice suppressed the proliferation of naïve responder $CD4^+$ T cells more vigorously than their counterparts from naïve controls (Fig. 6A and Tab. 2). For the lowest ratio of $CD4^+CD25^+CD103^+$ Tregs to responder cells (1:20) reflecting the *in vivo* situation, Tregs from the chronic phase of infection displayed the highest suppressive efficiency ($p<0.001$ compared to naïve and day 12 pi). The $CD4^+CD25^+CD103^+$ Tregs derived from infected animals were more efficient in mediating suppression than $CD4^+CD25^+CD103^-$ Tregs ($p<0.03$ for all tested ratios) (Fig. 6B and Tab. 2). As expected, both Treg subsets showed an anergic phenotype after polyclonal stimulation

by αCD3-antibodies (Fig. 6C). These data clearly indicate the strong *in vitro* suppressive capacity of $CD4^+CD25^+CD103^+$ Tregs derived from the chronic phase of infection with regard to activation and proliferation of $CD4^+$ T cells.

To evaluate whether IL-10 produced by Tregs contributes to their high suppressive capacity, suppression assays were performed with Treg cells form chronically infected donors in presence of αIL-10R antibody. This showed clearly that the *in vitro* suppression was independent of IL-10 (Fig 6D, E).

TABLE 2. Statistical analysis of suppressive efficiency of T_{reg} cells from naive and *H. polygyrus* infected mice.

ratio	$CD4^+$ $CD25^+$ $CD103^-$ [b]			$CD4^+$ $CD25^+$ $CD103^+$		
Treg: $CD4_{Resp}$ [a]	Naive vs. 12 dpi[c]	Naive vs. 28 dpi	12 vs. 28 dpi	Naive vs. 12 dpi	Naive vs. 28 dpi	12 vs. 28 dpi
1:20	ns	P<0.001	P<0.01	P<0.001	P<0.001	P<0.001
1:10	P<0.001	P<0.001	ns	P<0.001	P<0.001	ns
1:5	ns	P<0.01	ns	P<0.001	P<0.001	ns
1:2	P<0.001	ns	P<0.05	ns	ns	ns
1:1	ns	ns	ns	ns	ns	ns

[a] Ratio of T_{reg} cells to naive $CD4^+$ responders

[b] T_{reg} cell subsets analyzed for suppressive efficiency

[c] Statistical significance as determined by two-way ANOVA followed by Bonferroni post-test.

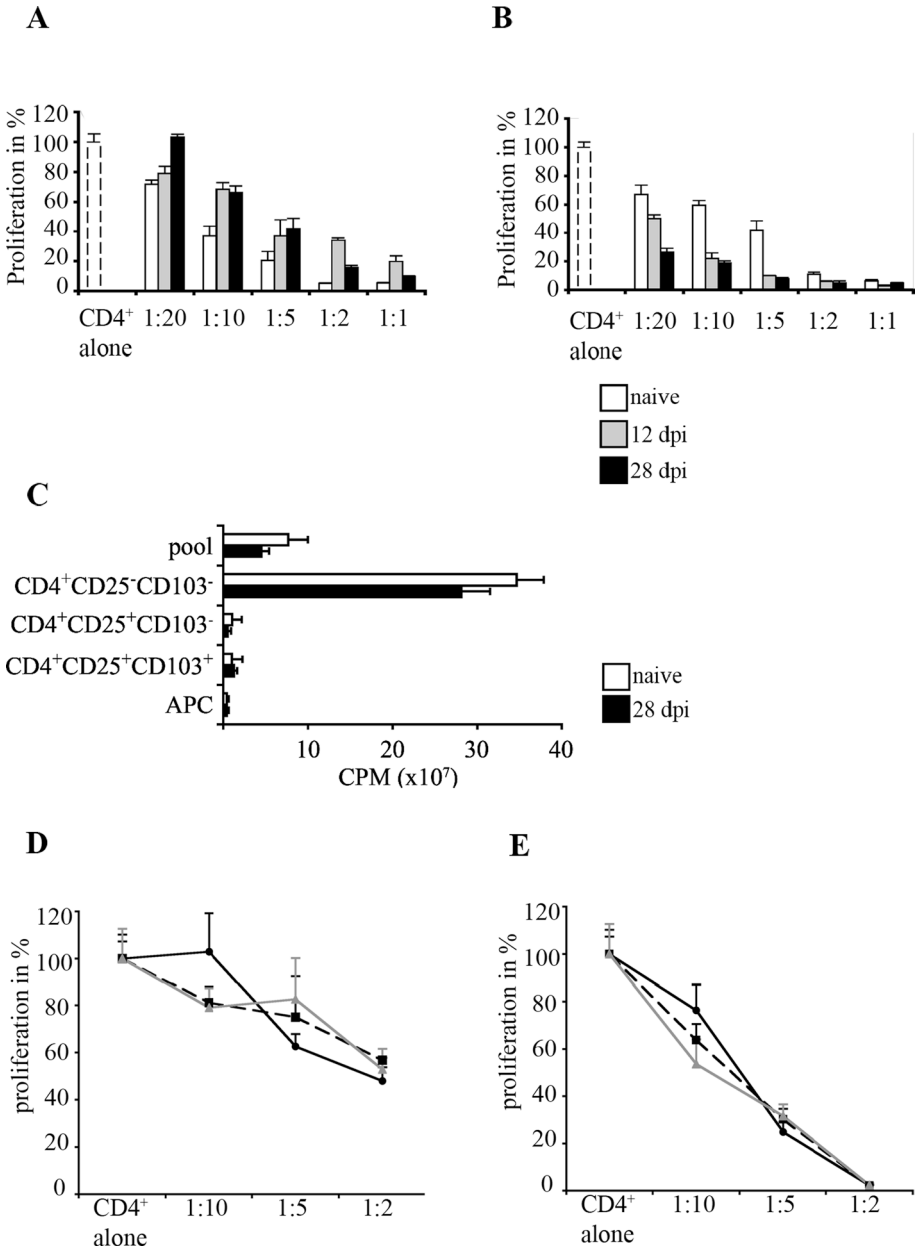

Fig. 6 Suppressive capacity and anergy of Tregs *in vitro*. $CD4^+CD25^+CD103^-$ and $CD4^+CD25^+CD103^+$ Tregs were purified from splenocytes and MLN cells of *H. polygyrus*-infected (12 and 28 dpi) and naïve mice. $CD4^+CD25^-CD103^-$ responder cells were isolated from naïve animals. (A) The suppressive capacity of $CD4^+CD25^+CD103^-$ Tregs and (B) of $CD4^+CD25^+CD103^+$ Tregs from naïve mice (open bars) and infected animals at the acute phase (12 dpi, grey bars) or chronic phase (28 dpi, black bars) of infection were analyzed. The ratio of Tregs and responder $CD4^+$ T cells is indicated. Proliferation of $CD4^+$ T cells after stimulation with αCD3 antibodies was detected by [^{3}H]-thymidine uptake. (C) Comparison of proliferative response of the $CD4^+$ subsets. The MLNC/splenocyte pool and sorted subsets were stimulated as indicated above. The background signal of irradiated APC is indicated. (D, E) Suppression by $CD4^+CD25^+CD103^-$ (D) and $CD4^+CD25^+CD103^+$ (E) Tregs from the chronic phase without (black lines) or with 10µg/ml αIL-10R ab (broken line) or total rat IgG (grey line) in culture medium. Mean + SEM of quintuple determinations is shown in all graphs. Data are representative for 2 to 3 experiments.

3.4 Cytokine production of complete and CD25/CD103-depleted MLNC.

MLNC of *H. polygyrus*-infected mice were isolated at different time points after infection and cultured in the presence of *H. polygyrus* antigen to analyze their cytokine profile. The Th2 key cytokines IL-4 and IL-13, as well as IL-10, were readily produced in high concentrations during the early phase of infection (6 dpi), accompanied by a weak IFN-γ response (Fig. 7A-D). Similar cytokine amounts were found during the acute phase (12 dpi), except for IFN-γ which was only marginally produced at this time point (Fig. 7A). At the chronic phase of infection (28 dpi) all cytokines analyzed were produced in lower amounts, especially IL-13 and IL-10. Although not reaching statistical significance, the trend of a lower cytokine production by MLNC from the chronic phase argues for a generally down-regulated parasite-specific response. Active TGF-β1 was detected only in very small amounts (<15pg/ml) irrespective of the time point (data not shown). Interestingly, depletion of cells with a mainly regulatory phenotype (displaying the surface markers CD25 and CD103) (Fig. 7E), resulted in drastic changes in cytokine production (Fig. 7A-D). First, in the early phase of infection (6 dpi), all cytokines analyzed were produced in lower amounts after depletion $CD25^+$ and $CD103^+$ cells. The finding of lower IL-4, IL-13, IL-10, TGF-β1 (not shown) and IFN-γ levels in cultures depleted of cells expressing CD25 and/or CD103 argues for depletion of not only Tregs, but also recently activated effector cells (Teff) at this early time point.

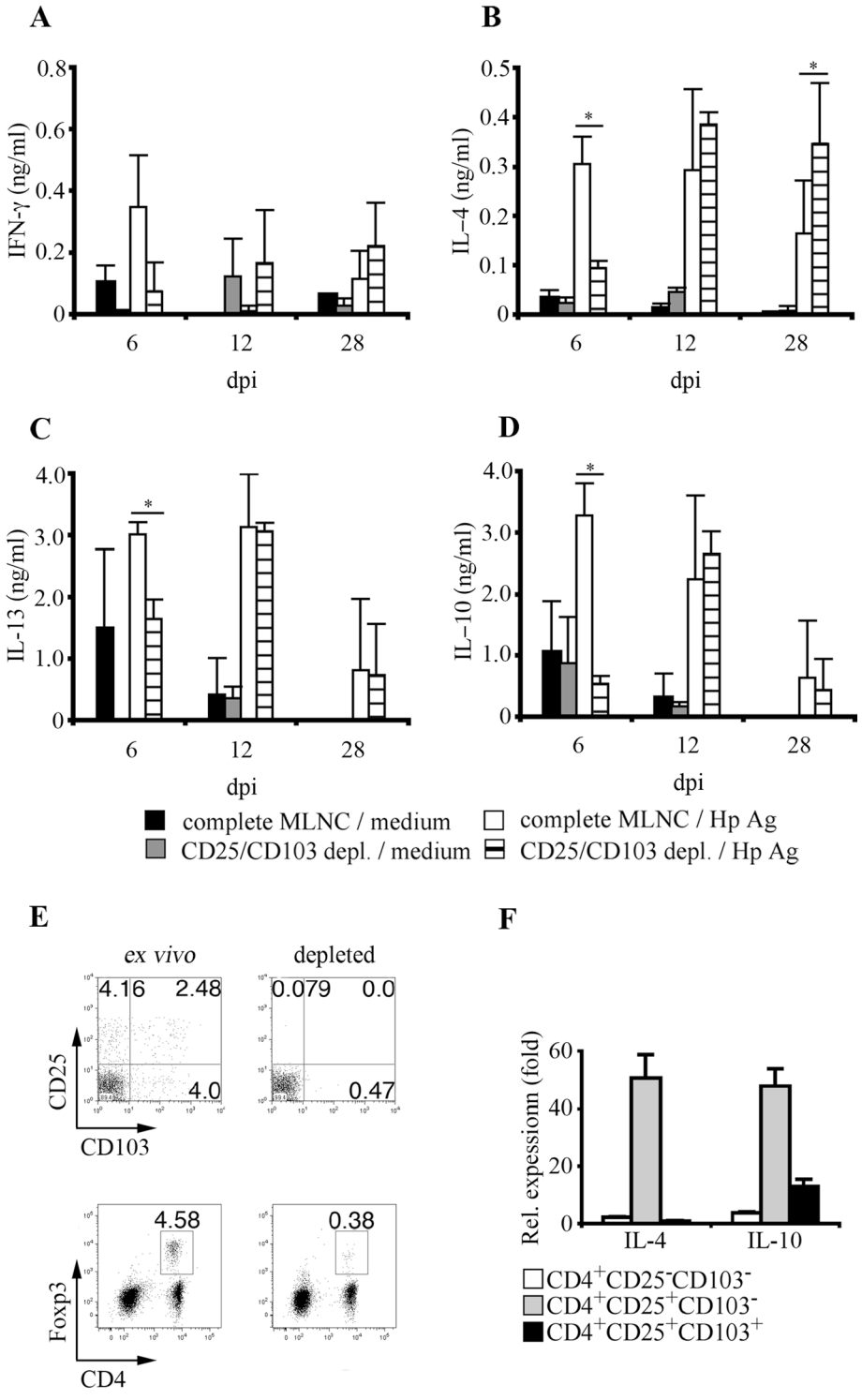

Fig. 7 Cytokine production of MLNC during *H. polygyrus* infection. The cytokine response of MLNC after restimulation with Hp Ag was analyzed at different time points pi. Complete MLNC (open bars) were compared to MLNC after depletion of CD25 and CD103 (hatched bars). IFN-γ (A), IL-4 (B), IL-13 (C) and IL-10 (D) production was analyzed. Mean + SD of three pools (two animals each) are shown. * Stat. sign. ($p < 0.05$). Data are representative for two independent experiments. (E) Efficiency of Treg depletion was determined by flow cytometry. (F) Relative expression of IL-4 and IL-10 mRNA by $CD4^+$ T cell subsets after coincubation with *H. polygyrus*-primed dendritic cells was assessed at day 6 pi. Mean + SD of three measurements is shown. Real-time PCR was performed by S. Pillai.

The presence of effector cells within the $CD4^+CD25^+CD103^-$ subset at 6 dpi was confirmed by detection of Foxp3 expression (showing a decline at 6 dpi, see Fig. 4A) and by analyzing IL-4 and IL-10 mRNA levels in different $CD4^+$ T cell subsets kept in cocultures with DC presenting *H. polygyrus* antigens (Fig. 7F). Here $CD4^+CD25^+CD103^-$ T cells expressed high amounts of IL-4- and IL-10-transcripts at this early time point, indicating the presence of Teff and Treg cells within this population. In contrast, the $CD4^+CD25^+CD103^+$ Treg population from the early infection exclusively expressed high levels of IL-10 (Fig. 7F). As the segregation of the IL-10 production to $Foxp^+$ and $Foxp3^-$ cells within the $CD4^+CD25^+CD103^-$ compartment was not analyzed, a contribution of recently activated effector cells to the IL-10 production at the early time point cannot be excluded.

Hence, the depletion of cells expressing CD25 and/or CD103 at the early time point removed a significant proportion of recently activated effector as well as regulatory T cells as seen by diminished production of the key Th2 cytokines IL-4 and IL-13 and IL-10 by remaining cells. The depletion had no effect with respect to the IL-10 production at later time points (Fig. 7D), arguing that cells other than Tregs may also represent important IL-10 sources during the acute and chronic phase. Interestingly, the IL-4 response in the chronic phase of infection was more vigorous after depletion, indicating a suppressive effect of Tregs on the Th2 response in the chronic phase of infection (Fig. 7B).

3.5 Cytokine production of $CD4^+$ T cell subsets in the chronic phase of infection.

The cytokine production of the $CD4^+$ T cell subsets was analyzed during the chronic phase of infection. Bone marrow derived dendritic cells were pretreated with *H. polygyrus* Ag (HpDC) and used as APC to stimulate the T cell subsets. Teff cells ($CD4^+CD25^-CD103^-$) released IL-4 and IL-13 in the presence of *H. polygyrus*-primed DC in contrast to Tregs ($CD4^+CD25^+CD103^-$ or $CD4^+CD25^+CD103^+$) (Fig. 8A, C). However, $CD4^+CD25^+CD103^+$ Tregs in particular released significant amounts of IL-10 (Fig. 8E). Incubation of the $CD4^+$ subsets with naïve DC (nDC) resulted in marginal release of cytokines by T cells, clearly showing the antigen-specificity of the cytokine response. Analysis of TGF-β1 revealed only marginal increases in cultures with HpDC compared to nDC. The highest levels were detected in cultures of conventional

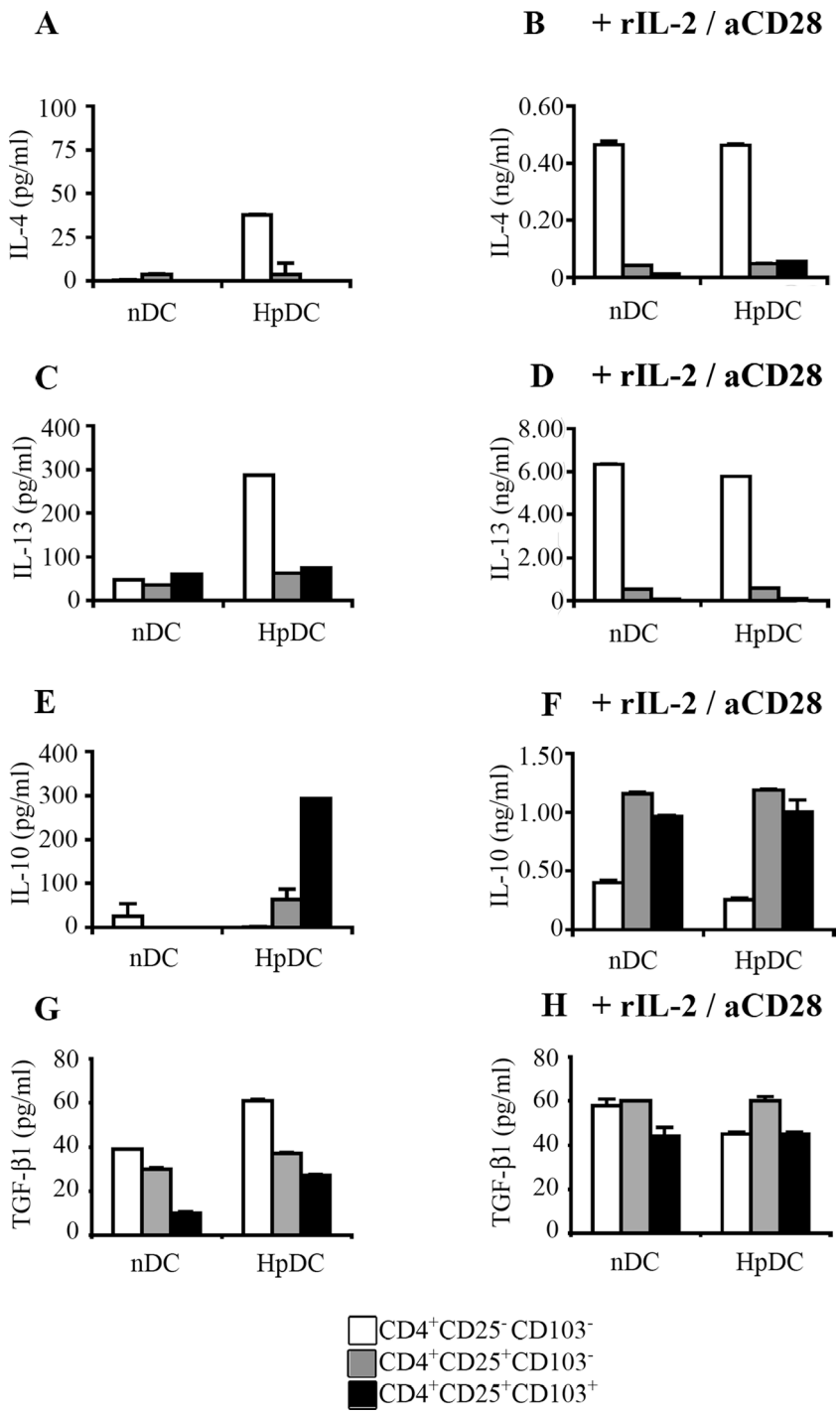

Fig. 8 Cytokine production by CD4$^+$ T cell subsets. CD4$^+$ T cells were isolated from pooled MLNC and splenocytes of 8 mice in the chronic phase of infection (28 dpi) according to the indicated surface marker expression. Cells were incubated for 72 h with naïve bmDC (nDC) or DC pretreated with *H. polygyrus* adult worm antigen (HpDC). Release of IL-4 (A, B), IL-13 (C, D), IL-10 (E, F) and active TGF-β1 (G, H) was detected by ELISA. Mean + SD of triplicate determinations is shown and data are representative for three independent experiments. Right panels show release of cytokines after addition of recombinant mouse IL-2 and αCD28 antibody to cultures. Mean values + SD of triplicate determinations of one of two independent experiments with similar results are shown.

$CD4^+CD25^-CD103^-$ cells, while both $CD4^+CD25^+CD103^-$ and $CD4^+CD25^+CD103^+$ produced lower amounts of active TGF-β1 (Fig. 8G). The generally lower cytokine levels in cultures of separated Teff cells compared to complete MLNC cultures (Fig. 7) is probably due to less $CD4^+$ T cells present in DC cocultures than in preparations of whole MLNC, and to other cytokine producing cells present in MLNC cultures, such as basophils, mast cells and eosinophils (76).

While only $CD103^+$ Tregs produced IL-10 in an antigen-specific manner, both Treg subsets produced high amounts of IL-10 when optimized conditions were provided by addition of exogenous IL-2 and an enhanced costimulation through αCD28 antibodies (Fig. 8F). Similarly, IL-4 and IL-13 were produced in much higher amounts almost exclusively by $CD4^+CD25^-CD103^-$ Teff cells after providing enhanced costimulation (Fig. 8B, D). TGF-β1 production was only marginally affected (Fig. 8H). None of the $CD4^+$ T cell subsets from *H. polygyrus*-infected mice secreted relevant amounts of the Th1 cytokine IFN-γ (data not shown). Hence, a dominant, parasite-specific Th2 response is exhibited by $CD4^+$ effector cells from the chronic phase of infection, while effector/memory-like $CD4^+CD25^+CD103^+$ Tregs produce IL-10 in response to *H. polygyrus* antigen.

3.6 Adoptive transfer of CD4+ T cells from *H. polygyrus*-infected mice.

To analyze the function of different $CD4^+$ T cell subsets *in vivo*, adoptive transfers of $CD4^+$ Teff cells and Treg subsets derived from chronically *H. polygyrus*-infected mice (28 dpi) were performed. Teff cells ($CD4^+CD25^-CD103^-$) and $CD4^+$ T cells with a regulatory phenotype, namely $CD4^+CD25^+CD103^-$ and $CD4^+CD25^+CD103^+$ T cells, were transferred to naïve recipients. Control animals received PBS only. One day after transfer, recipients were infected with a defined dose of larvae. The purity of Teff cells was >98% for expression of $CD4^+$ with <3% remaining $CD25^+$ and/or $CD103^+$ cells, whereas the Treg compartments were >90% $CD4^+CD25^+CD103^-$ cells and >89% $CD4^+CD25^+CD103^+$ cells, respectively (Fig. 9A).

Four weeks after infection, adult worm burdens of recipients and control animals were assessed. A significant reduction of 43.7% ($p<0.008$) of worm burden was detected in animals receiving Teff cells ($CD4^+CD25^-CD103^-$) compared to the PBS control group (Fig. 9B). In contrast, transfer of $CD4^+CD25^-CD103^-$ T cells from naive mice had no influence on the worm burden (data not shown), indicating that the reduction of worm burden was due to parasite-

specific T_{eff} cells. The protective role could be solely ascribed to $CD4^+$ T cells, as the transfer of $CD4^-$ cells had no effect on worm burden (data not shown).

Animals receiving $CD4^+CD25^+CD103^-$ Tregs (comprising >90% $Foxp3^+$ cells) had worm burdens comparable to the PBS control. Similarly, the transfer of $CD4^+CD25^+CD103^+$ Tregs (comprising >95% $Foxp3^+$ cells) did not result in significant changes in worm burden. In contrast, transfer of a heterogenous T cell population containing Tregs and Teff cells, namely $CD4^+CD103^+$ T cells (with a purity of >95% $CD4^+$ cells and >85% $CD103^+$ cells but

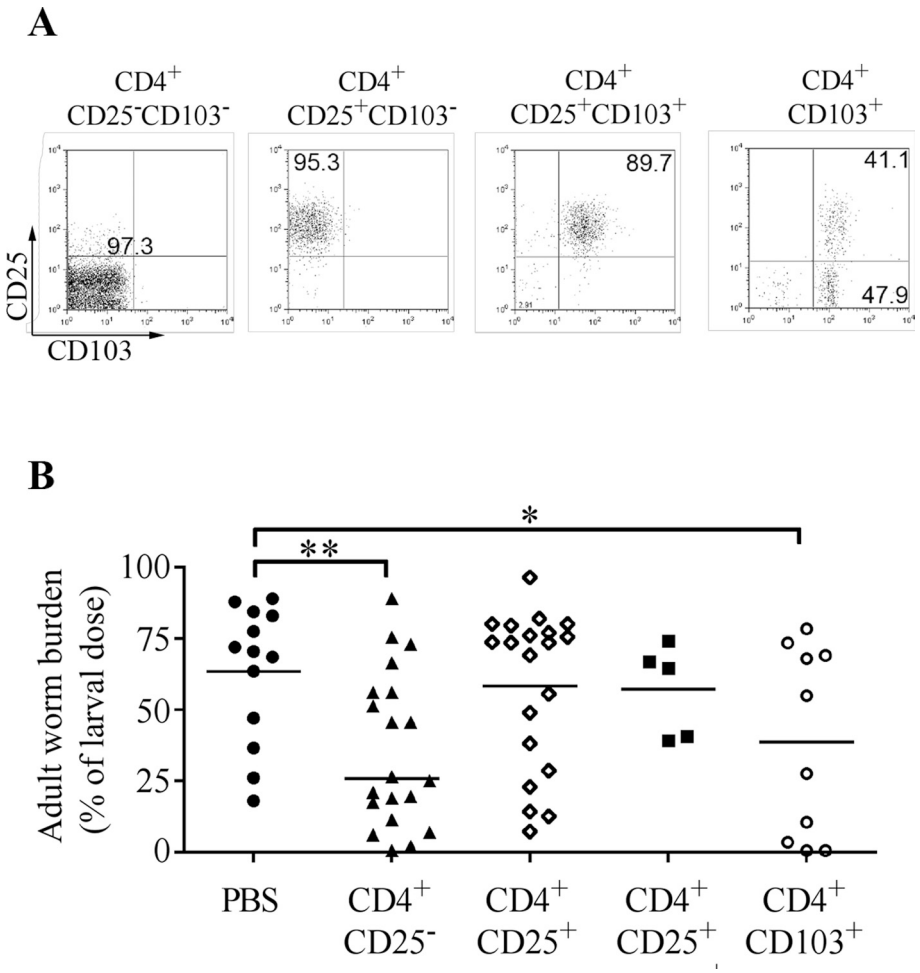

Fig. 9 Influence of adoptive $CD4^+$ T cell transfer on adult worm burden. 5 x 10^5 T cells of the indicated subtypes obtained from MLN and spleen of chronically *H. polygyrus*-infected mice were transferred to recipients that were subsequently infected with *H. polygyrus* larvae. Control animals received PBS only. (A) Purity of transferred cells as determined by flow cytometry. Representative data from one of three independent experiments are shown. (B) Adult worm burden in recipients 28 dpi. Worm counts are shown as percentage of applied larvae. Group sizes varied between 5 to 20 animals. Data originate from three individual experiments. Individual worm counts and median are shown. Statistical significance (*) $p < 0.05$; (**) $p < 0.01$.

comprising only about 70% $Foxp3^+$ cells) led to a significant decrease of worm burden ($p<0.03$) comparable to pure $CD4^+$ Teff cells (Fig. 9B).

Hence, $CD4^+$ Teff cells from the chronic phase of a *H. polygyrus* infection transferred protection to naïve recipients, in contrast to naturally occurring or effector/memory-like Tregs that had no intrinsic effect on worm development. Furthermore, transfer of a mixed T cell population comprising effector and regulatory T cells showed that Tregs were not efficient in suppressing the protective effect mediated by Teff cells.

3.7 Tracking of transferred cells *in vivo*

To gather information about the migratory behaviour and the survival of applied $CD4^+$ T cell subsets, total $CD4^+$ cells derived from MLN and spleen of chronically infected GFP-expressing donors were transferred. To facilitate the reanalysis, $1x10^7$ cells per recipient were transferred. Cell survival and migration was assessed according to two different protocols concerning the application and the setup of infection: first, cell application directly to the blood stream via the tail vein versus transfer to the peritoneal cavity, and second, transfer to naïve recipients infected the following day versus animals at the early acute phase (6dpi) of infection. None of the parameters revealed differences with respect to migration pattern or survival rate (data not shown).

Six days after transfer, spleen, MLN as well as intraepithelial (IEL) and lamina propria (LPL) lymphocytes from the small intestine were analyzed (Fig. 10A). Transferred cells were traced in all organs analyzed with no significant accumulation in any of the compartments. The slightly elevated numbers of GFP^+ cells within MLNC and LPL compared to splenocytes and IEL merely reflect the higher $CD4^+$ T cell numbers present in lymph nodes and the lamina propria (Fig. 10C). As expected, intraperitoneal cell application led to higher proportions of transferred cells within the peritoneal exudates cells. Interestingly, a proportion similar to that found in lymphatic organs and the small intestinal compartments could be detected after cell transfer to the blood stream, showing that cells actively migrate to this site (data not shown).

The reanalysis after transfer revealed the presence of CD25 and CD103 expressing cells within the transferred population (Fig. 10B, upper panel), albeit at lower levels than seen within the endogenous lymphocyte population. The $CD4^+CD25^+CD103^-$ and $CD4^+CD25^+CD103^+$ compartments clearly contained Foxp3 expressing cells, however again at

lower ratios than seen in the endogenous populations (Fig. 10B, lower panel). These data show that Tregs survive the transfer and can be detected at least one week later, however in decreased proportions. Whether these data indicate a lower survival rate of transferred Tregs or reflect an artefact due to low GFP^+ cell numbers reanalyzed is not clear.

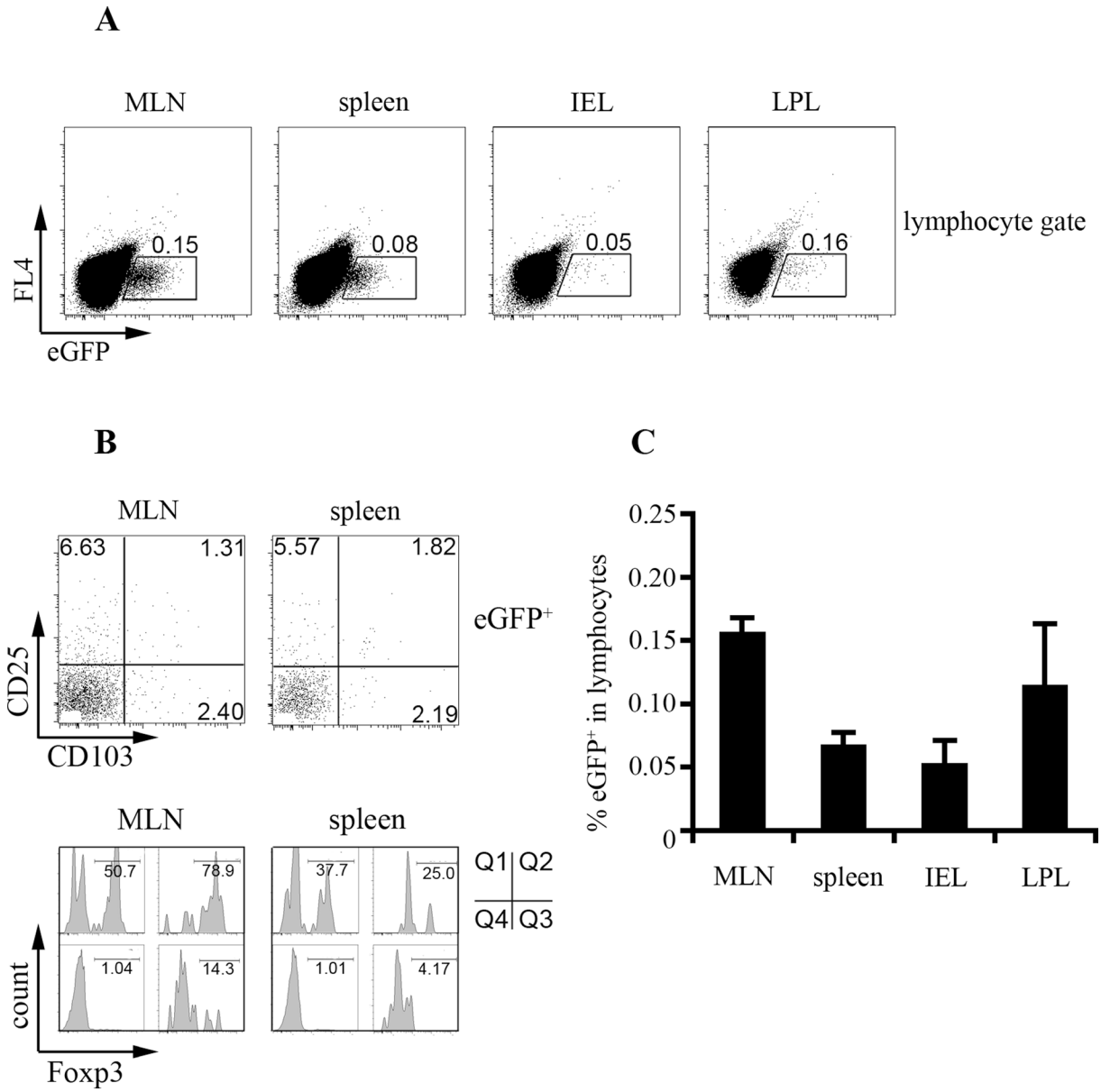

Fig. 10 Tracing of transferred cells in C57BL/6 mice in a short-term approach. 1x10^7 $CD4^+$ cells from chronically infected eGFP-transgenic donors were transferred to naïve mice. Recipients were infected the following day and dissected 6 days post transfer. (A) Detection of $eGFP^+$ cells in MLN, spleen, IEL and LPL. Values indicate the proportion of GFP^+ cells within the lymphocyte gate. (B) Treg analysis within transferred cells according to expression of CD25 and CD103 (upper panel) and Foxp3-detection within the $CD4^+$ subsets (lower panel). Values indicate the percentage of Foxp3 expressing cells within the four $CD4^+$ populations. Exemplary plots for a group of 4 animals are shown. The experiment was performed twice with similar results. (C) Percentage of transferred cells within lymphocytes from different compartments. Mean + SEM of 4 animals is shown. Two experiments with similar results were performed.

Unfortunately, due to limited cell numbers obtained from the intestine, no detailed subset-analysis was possible for the LPL and IEL compartment, leaving the question unanswered, whether Tregs might preferentially migrate to the site of infection.

To investigate the long term survival and development of transferred $CD4^+$ cells, recipients were analyzed 25 days post transfer. As previous experiments had revealed difficulties in detecting the GFP^+ signal in fixed cells, the analysis was performed with

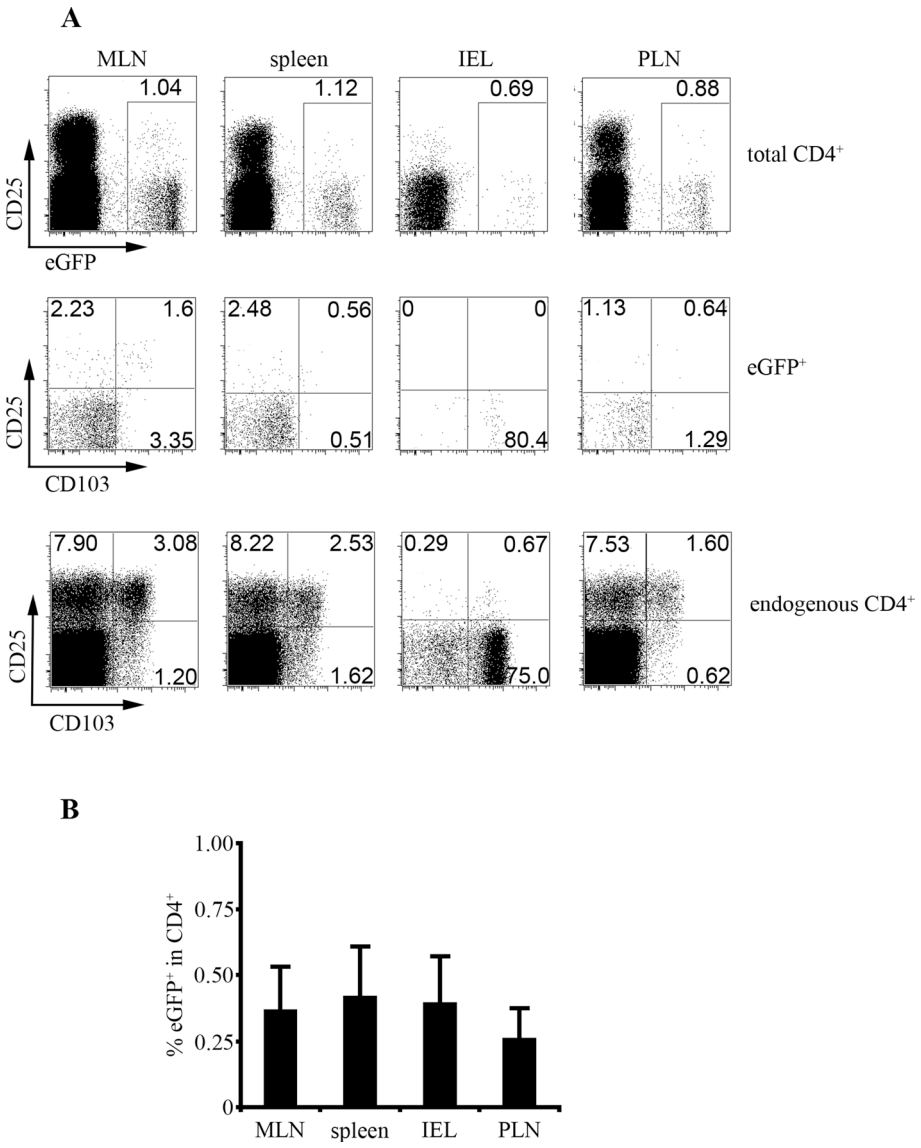

Fig. 11 Tracing of transferred cells in a long-term approach. The experiment was performed as described in Fig. 10 and recipients were dissected 25 days post transfer. (A) Detection of GFP^+ cells in MLN, spleen, IEL and peripheral lymph nodes (PLN). Values indicate the proportion of GFP^+ cells within the $CD4^+$ gate (upper panel). Mid panel: detection of CD25 and CD103 on transferred cells. Lower panel: Expression of CD25 and CD103 by endogenous $CD4^+$ cells. (C) Percentage of transferred cells within $CD4^+$ cells from different compartments. Mean + SEM of 5 animals is shown.

live cells in this long-term approach. Therefore, the GFP-signal appears much brighter (Fig. 11A, upper panel). While the intracellular detection of Foxp3 was impossible when working with live cells, the expression of CD25 and CD103 was analyzed. Unexpectedly, very low proportions of cells expressing the regulatory markers were detected (Fig. 11A, mid panel), especially obvious when compared to the corresponding endogenous $CD4^+$ compartment (Fig. 11A, lower panel).

Only GFP^+ cells derived from the intestinal epithelium expressed CD103 with frequencies of 70-80%, however this reflected the finding on endogenous T cells and virtually no $CD103^+$ cells co-expressed CD25. This CD103 expression pattern of $CD4^+$ cells is in accordance with earlier descriptions for the intestinal T cell compartment (32, 108).

The dissemination of GFP^+CD4^+ cells showed a similar pattern to what was seen after short-term transfers (Fig 11B, compare to 10C). The slightly higher levels of GFP^+ expressing cells detected in all compartments after the long term approach is due to the gating on $CD4^+$ T cells, which was not possible analyzing the short-term experiments due to an unreliable CD4 staining in the latter. These data show, that the differences concerning the frequencies of transferred cells within the different compartments seen in short-term experiments (Fig. 10C) reflect the different proportions of $CD4^+$ cells present. Proper gating on $CD4^+$ cells in the long-term experiment revealed a similar proportion of transferred cells within all compartments analyzed, including peripheral lymph nodes (PLN) presumably unaffected by the infection (Fig. 11B).

Interestingly, homing experiments with *in vitro* generated Th1 and Th2 CD4 cells transferred to subsequently or readily infected animals showed no overt accumulation of these cells in any intestinal compartment, neither in response to primary nor to secondary *H. polygyrus* infection (U. Hoffmann, personal communication). This raises the question whether the parasite might interfere with efficient T cell homing to the site of infection (U. Hoffmann, personal communication).

3.8 Distribution of antigen-specific $CD4^+$ T cells and their cytokine production.

To assess the distribution of *H. polygyrus*-specific $CD4^+$ T cells within the Teff and Treg populations, the expression of CD154 as a marker of antigen-specific $CD4^+$ T cell activation (71,110) was assessed. As the direct detection of CD154 *ex vivo* is not possible, an optimized *in vitro* protocol was applied (110). Comparison of $CD4^+$ T cells from spleen and MLN showed that in both sites about 0.2% (MLN: 0.233 $\pm$ 0.036, naïve vs. infected $p<0.001$; spleen: 0.246 $\pm$ 0.040, naïve vs. infected $p<0.0003$) of $CD4^+CD103^-$ Teff cells expressed CD154 when restimulated with adult worm antigen *in vitro* (Fig. 12A, B). By additional detection of CD103 and Foxp3, it was possible to distinguish between CD154-expression on Teff and Treg cells.

Interestingly, when analyzing the level of CD154 on $CD103^+$ cells not co-expressing Foxp3 and therefore not of regulatory phenotype, a clearly higher percentages of antigen-specific cells was detected as compared to $CD4^+CD103^-$ Teff cells in MLN (1.701% $\pm$ 0.306, $CD4^+CD103^-$ vs. $CD4^+CD103^+$ $p<0.0006$) and spleen (0.586% $\pm$ 0.144, $CD4^+CD103^-$ vs. $CD4^+CD103^+$ $p<0.01$) (Fig. 12A, C) of infected animals. In contrast, only low levels of CD154 expression were detected on $Foxp3^+$ T_{reg} cells from MLN (<0.03%) and spleen (<0.05%) (data not shown). This indicates that mice adoptively transferred with T cells sorted for co-expression of CD4 and CD103 (irrespective of CD25-expression) (Fig. 9B) received a highly enriched T_{reg} population which still contained a low number of antigen-specific effector cells not expressing Foxp3. The percentage of antigen-specific effectors within the transferred $CD4^+CD103^+$ cell compartment was as low as 0.2% of all cells, comparable to the values for antigen-specific cells within the $CD4^+CD103^-$ effector compartment. These $CD103^+$ effectors may have led to protection even though a large number of T_{reg} cells was cotransferred, arguing for an insufficient capacity of the T_{reg} cells to control the antigen-specific effectors.

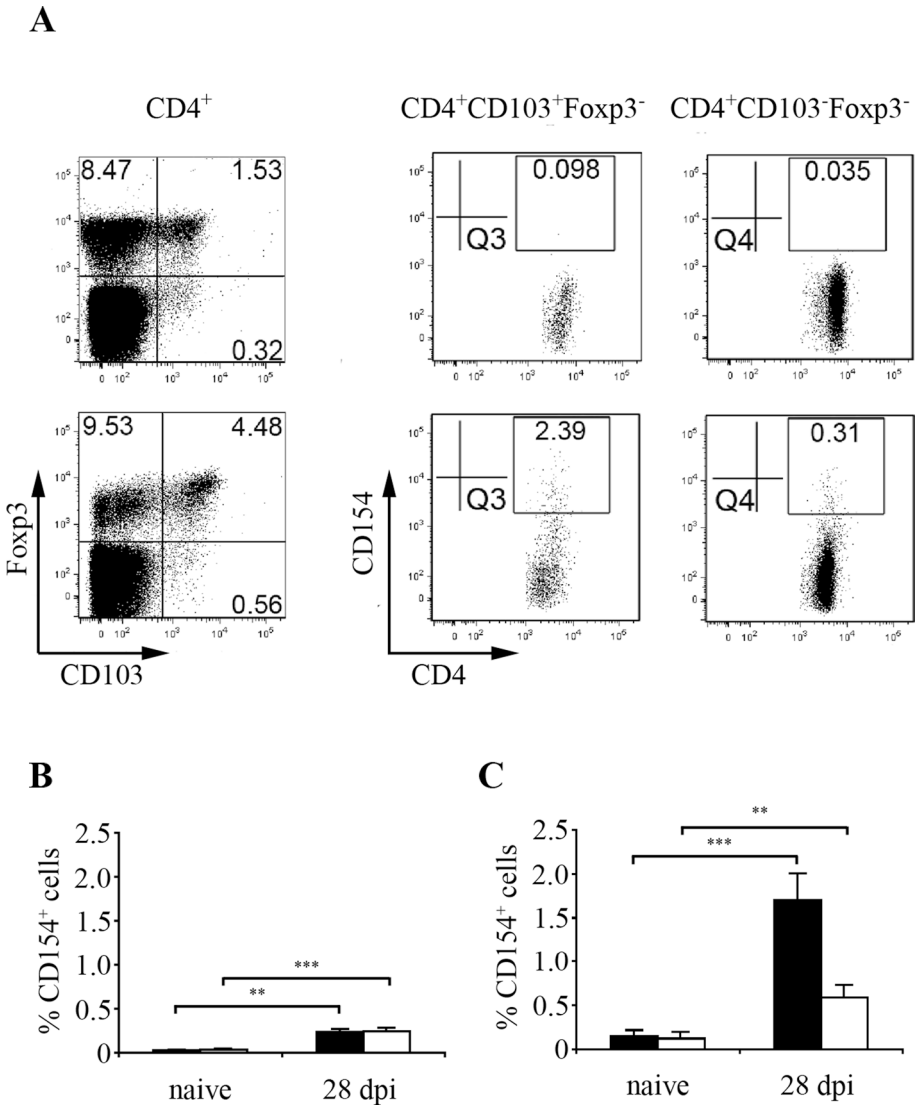

Fig. 12 Distribution of antigen-specific CD4$^+$ T cells. (A) Exemplary FACS plots of MLNC from naive (upper row) and *H. polygyrus* infected mice at day 28 pi (lower row). Cells were restimulated with *H. polygyrus* antigen and stained for CD4, CD154, CD103 and Foxp3. CD4$^+$ cells were plotted for expression of CD103 and Foxp3 (left panels). Mid panels: CD154 expression by CD4$^+$CD103$^+$Foxp3$^-$ Teff cells. Right panels: CD154-expression by CD4$^+$CD103$^-$Foxp3$^-$ Teff. Frequencies of CD154 expression by CD4$^+$CD103$^-$Foxp3$^-$ (B) and CD4$^+$CD103$^+$Foxp3$^-$ (C) Teff cells of MLN (black bars) and spleen (open bars). Data are derived from 8 infected and 7 naïve mice. Mean + SEM is shown. Data are representative for three independent experiments.

Next the cytokine production of the antigen-specific CD154$^+$CD4$^+$ T cells was determined (the intracellular cytokine analysis presented here was performed by Dennis Kirchoff, DRFZ, Berlin). Investigation of IL-4, IL-13, IL-10 and IFN-γ production after *in vitro* restimulation (Fig. 13A) revealed a dominant Th2 response in CD154$^+$CD4$^+$ T cells of chronically infected mice, characterized by high expression levels of IL-4 (24.55% ± 1.32), IL-13 (10.48% ± 1.99) or both (10.26% ± 1.02) (Fig. 13A, B). Only low levels of IFN-γ-producing cells were detected in the antigen-specific T cell compartment (4.47% ± 0.82), and frequencies of IL-10-producing cells

were hardly distinguishable from unspecific background (1.60% ± 0.37) (Fig. 13A, C). These data again indicate that the lower worm burden in recipients receiving CD103-expressing $CD4^+$ T cells might be due to co-transfer of antigen-specific effector cells that were able to produce IL-4 and IL-13, thereby mediating worm expulsion.

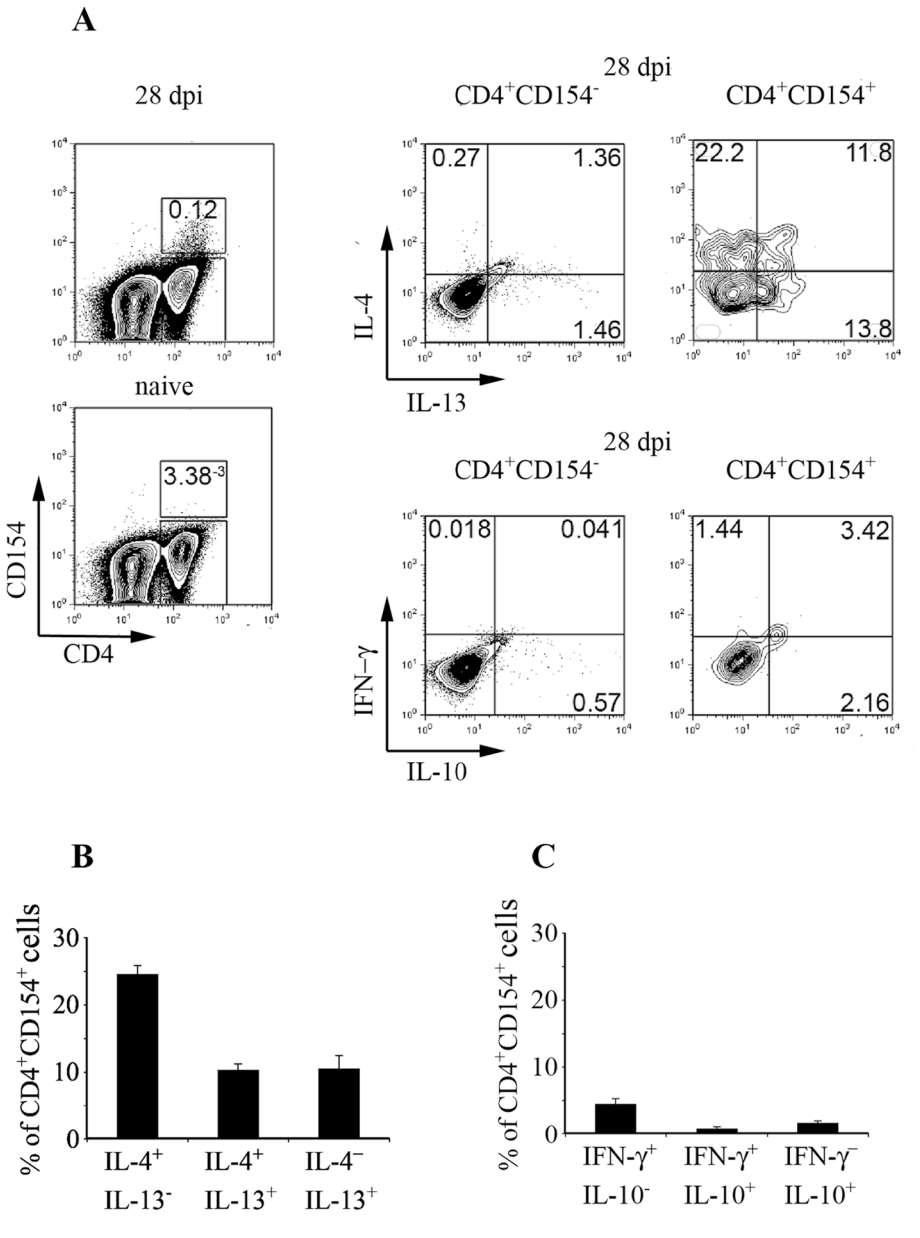

Fig. 13 Intracellular detection of cytokines within restimulated $CD4^+$ T cells from spleens (28 dpi). (A) Cells were gated for expression of CD4 and CD154 (left panel) and IL-4 and IL-13 (upper row) or IFNγ and IL-10 (lower row) were determined in $CD4^+CD154^-$ cells (center panels) and $CD4^+CD154^+$ cells (right panels). Cytometry plots shown are representative for a group of 8 infected mice. (B) IL-4/IL-13 and IFN-γ/IL-10 (C) response in $CD4^+CD154^+$ cells after restimulation with *H. polygyrus* antigen. Mean + SEM of 8 animals is shown. * Statistical significance comparing cells from naïve and infected animals as determined by Mann-Whitney test, (**) $p < 0.01$; (***) $p < 0.001$.

3.9 Application of filarial cystatin in a murine model of colitis

A second project of this PhD work was to investigate the effect of a defined immunomodulatory parasite component, the filarial cystatin Av17, in a model of chemically induced colitis. In this model intestinal inflammation is induced by feeding the chemical dextran sodium sulfate, which is toxic to colonic epithelial cells, thereby leading to mucus layer destruction and disruption of the barrier function of the epithelium, followed by tissue remodelling and infiltration of immune cells due to increased contact with luminal contents.

As a first approach, mice were fed for one week with the chemical in drinking water to induce acute colitis. Groups of mice were treated with *E.coli*-expressed recombinant Av17 applied intraperitoneally in four 20µg doses every second day (Fig. 14). *E.coli*-expressed recombinant DHFR (mouse dehydrofolate reductase) served as control protein. Mice were allowed to recover for approximately 20 hours after seven days. Then the colon was removed and sections were prepared for histopathological analysis (Fig. 15).

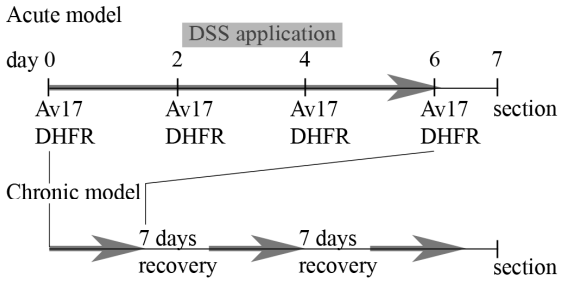

Fig. 14 Acute and chronic colitis model. Mice were fed with 2.5% of DSS in drinking water for 7 days and then kept DSS-free for approx. 20h before section (acute model) or at least 7 days before the next DSS cycle (chronic model). Along with DSS-feeding, Av17 or DHFR was applied 4 times ip. In the chronic model, some animals were left untreated during the first DSS cycle (therapeutic), while others were treated from the start (preventive).

Cross sections of all animals were scored according to a system based on the size of epithelial erosions, goblet cell loss and size of cellular infiltrates as well as colonic areas afflicted (transmural infiltrates or cellular accumulations restricted to mucosa and submucosa). While animals receiving DSS and sham-treated with protein application buffer showed severe signs of inflammation with gross epithelial erosions, loss of goblet cells and transmural mononuclear cell infiltrates, animals treated with Av17 showed significantly lower signs of inflammation with reduced thickening of the colon wall, wide areas of healthy epithelial appearance

with goblet cells present and only focal, superficial erosions. In contrast, animals treated with the control protein DHFR resembled to the DSS group (Fig. 15, 16).

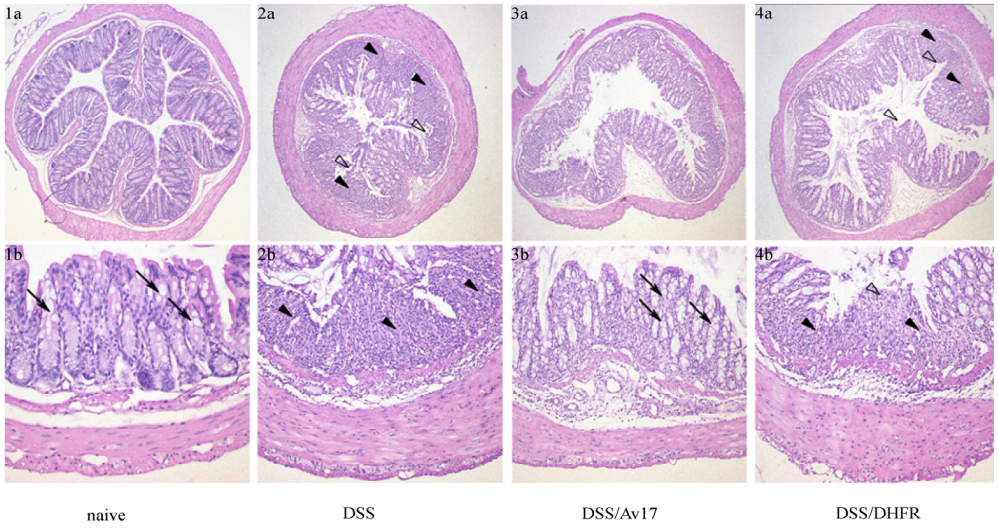

Fig.15 Histological appearance of colon cross sections. H/E stained sections of the colon of animals from the acute colitis model are shown. Upper row (a): 40x magnification, lower row (b): 200x magnification. Sections of an untreated (1), DSS and protein application buffer (2), DSS/Av17 (3) and DSS/DHFR (4) treated animal are shown. Black arrows: goblet cells, white arrow heads: erosions; black arrow heads: dense cellular infiltrates.

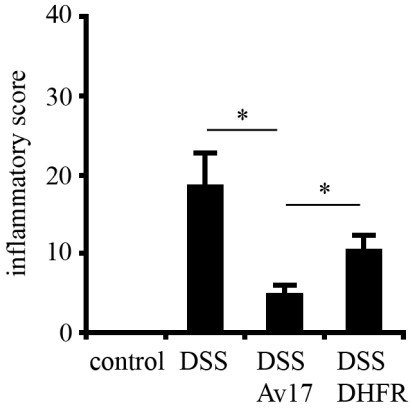

Fig. 16 Av17-treatment impairs acute colitic response to DSS. Mice were fed for 7 days with DSS in drinking water to induce acute colitis. Treatment with four doses of Av17 significantly reduced the inflammatory score of mice, while the recombinant control protein DHFR showed no significant effect. Control animals were kept on DSS-free drinking water and were left untreated.* Statistical significance ($p < 0.05$). Combined data of two experiments with 4-5 animals per group are shown.

As Av17-treatment showed a protective effect on the short-term acute colitis model, the next step was the application of the parasite component during the chronic model based on repeated DSS-feeding cycles (Fig. 14). To compare the preventive and therapeutic effect of Av17 on colitis, animals were either treated with the protein throughout all DSS-feeding cycles or left untreated during the first inflammation-inducing cycle. However, during several chronic model experiments conducted with varying doses of DSS, high losses of animals had to be faced. Around 50 percent of the animals died in all approaches before the treatment cycles were completed and the effect of Av17 treatment was not as clear as in the acute model. Still, an ameliorating affect of Av17 was visible in one approach, although no statistical analysis was possible due to high animal losses. Again, this effect was clearly distinct from minor changes induced by the control protein. Of note, only the preventive model of Av17 treatment revealed a clear decrease in inflammatory signs, while the therapeutic approach revealed no overt effect of Av17 (Fig. 17).

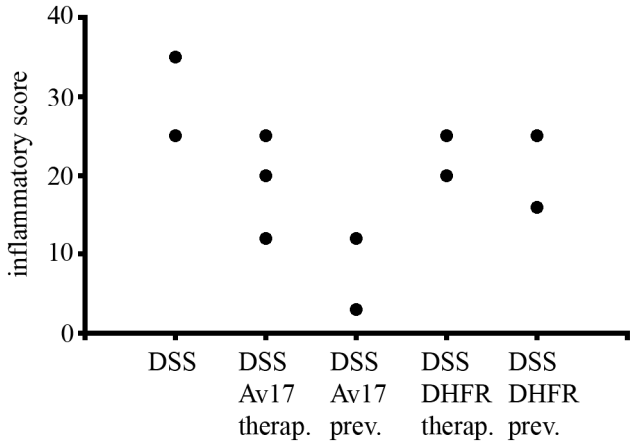

Fig. 17 Effect on chronic colitis by Av17-treatment. Mice received three one-week cycles of DSS-feeding and were allowed to recover for at least 7 days between DSS cycles. Animals were treated with four doses of Av17 or DHFR in the second and third DSS cycle (therapeutic model) or in all three DSS cycles (preventive model). The preventive approach revealed an ameliorating effect of Av17-treatment on inflammation.

Besides the histopathologic scoring, all experiments included a second readout on the basis of the IFN-γ response. To that end, lamina propria lymphocytes were isolated from the colon and restimulated polyclonally with phytohemagglutinin *in vitro*. As T helper cells are especially addressed during the long-term DSS treatment in the chronic model, this cytokine readout may be helpful to make out differences between heavily and slightly colitic animals. However, none of the experiments revealed consistent differences between treatment

groups. In most cases, even animals from the DSS control group reaching high inflammatory scores during the acute and chronic model showed inconvenient IFN-γ levels. This was probably due to insufficient viability of the isolated cells. In addition, IL-4 and IL-10 measured in parallel showed similar patterns, arguing for the unreliability of the cytokine read-out data (not shown).

4 Discussion

This work investigated the role of different $CD4^+$ T cell subsets as well as the dynamics within regulatory T cell populations in response to infection with the gastrointestinal (GI) nematode *H. polygyrus*. This parasite is generally used as a model to study immune responses during chronic GI infections and protective mechanisms against reinfection. The key questions addressed in this work are whether an infection with this nematode has an effect on Tregs and whether T cells from infected animals interfere with the outcome of a primary infection following adoptive transfer.

The data presented here provide evidence for the following traits of Teff/Treg interaction: First, Teff cells that arise during a primary *H. polygyrus* infection mediate protection. Second, Teff cells persist in chronically infected mice, despite a strong cellular suppression (52, 189, 200). Third, less than 1% of $CD4^+$ Teff cells are parasite-specific cells according to expression of CD154 and the release of IL-4 and IL-13 in response to parasite antigen. Fourth, Tregs represent an important source of IL-10 during infection. And fifth, elevated numbers of effector/memory-like Tregs persist in lymphatic organs until the chronic phase and Tregs derived from infected animals show an augmented suppressive capacity *in vitro*.

4.1 Dynamics and phenotype of Tregs during *H. polygyrus*-infection

Peripheral Tregs are not only needed to control autoreactive T cells escaping negative selection in the thymus, but they are also active in controlling immune responses against pathogens. (25, 94, 118, 181, 149, 194). Studies with animal models or parasite-infected patients showed that Tregs are needed to control immunopathology (15, 19, 29, 92, 122, 162), but on the other hand may favour parasite survival (23, 94, 228, 246, 256). Clearly, the delicate balance between protective immune responses and uncontrolled pathology is a field of Treg action, but these cells may also be required to establish immunity (23).

It has been recognized for long, that chronic helminth infections lead to upregulation of anti-inflammtory cytokines such as TGF-β and IL-10 (51, 68, 92, 109, 135, 186, 201). Tregs are a source for both and their induction and outgrowth is favoured by these cytokines (12, 35, 38,

41, 62, 72, 126, 260). In addition, worm infections are described to down-modulate APC functions, which in turn might also lead to Treg induction, e.g. by immature dendritic cells.

The large body of recent data demonstrating a role of Tregs in a broad spectrum of infections suggests that these cells are addressed in virtually all settings of inflammation or infection in immunocompetent individuals. This probably reflects a common circuit against overwhelming immunopathology. However, to date it is not clear, whether certain pathogens may specifically exploit circuits of Treg activation or induction.

Clear hints for immunosuppression during infection with *H*. *polygyrus* come from the kinetic of the parasite specific Th2 response. The present work and others show that the initial IL-4/IL-13 response peaking between the second and third week after infection is downmodulated in the chronic phase with persisting and reproducing adult worms (68). The secretion of IL-10 reflects this pattern. However, others have shown increased levels of TGF-β1 also in the chronic phase of infection (52, 53) and this cytokine is known to suppress both Th2 and Th1 responses (125). Furthermore, the induction of a regulatory cytokine response by $CD4^+$ cells in the murine intestine is described as protective against chemically induced colitis (56, 201), and intestinal $CD8^+$ cells from infected animals bear suppressive effects on other T cells (150). *H. polygyrus* infection has been shown to interfere with the development of allergic airway disorders and this effect is partially dependent on $CD4^+CD25^+$ T cells (252). The infection also prevents an allergic response to dietary antigens in an IL-10 dependent manner (14).

Taken together, these findings argue for *H. polygyrus* addressing regulatory circuits of the host's immune system. Hence, this work analyzed the changes occurring among the regulatory $CD4^+$ T cell compartment with respect to number, phenotype and activity of Tregs alongside a primary *H. polygyrus* infection. The dynamics of naïve- and effector/memory-like Tregs in lymphatic organs were one major point of interest and analyzed according to expression of CD25, CD103 and Foxp3 within the $CD4^+$ T cell population.

The analysis of Treg frequencies relying on the most definite marker Foxp3 revealed that the proportion of Tregs within $CD4^+$ cells remained unchanged after infection. However, a disproportional and permanent increase of the frequency of $CD4^+CD25^+Foxp3^+$ cells coexpressing the α-subunit (CD103) of the integrin $\alpha_E\beta_7$ was detected. This finding was accompanied

by a decrease in the proportion of $CD4^+CD25^+Foxp3^+CD103^-$ cells. These changes occurred early in the MLN draining the site of infection and were detected systemically with a short delay. The increased proportion of $CD103^+$ Tregs was stable during persistent infection.

The finding of an increased proportion of $Foxp3^+$ cells expressing CD103 argues for a qualitative difference of Tregs in response to infection with *H. polygyrus.* This integrin is described as a marker for effector/memory-like Tregs with particular suppressive capacity and migratory behaviour, specialized for crosstalk with epithelial compartments (97, 124, 205). Thus, $CD103^+$ cells do not represent a distinct regulatory lineage. CD103 recognizes epithelial cadherin, thereby enabling cellular retention in compartments such as the skin, lung and gut mucosa, and its expression is positively regulated by TGF-β (31, 188). Studies on CD103 expression by regulatory T cells during infection showed, that the integrin is upregulated shortly before or after migration of Tregs to epithelial sites and is needed for retention at inflamed tissue (216). *Leishmania* species have evolved mechanisms to induce TGF-β production by infected host cells (exclusively macrophages). This might explain the finding of CD103 upregulation and expansion of Tregs, as seen at the inflamed site in such model infections (11, 216).

Studies on the expression of adhesion molecules and chemokine receptors and the migratory behaviour of $CD103^+$ and $CD103^-$ regulatory subsets revealed that the former have the peculiar ability to migrate to inflamed sites, while the latter recirculate through lymphoid compartments (98). More recently it has been shown that $CD103^+Foxp3^+$ cells originate from both naïve-like ($CD4^+CD25^+CD103^-$) natural Tregs and naïve T cells (206). Interestingly, this experience-driven development of effector/memory-like Tregs was shown under the regimen of oral antigen application, which is known to induce oral tolerance (37, 193).

While an increased Foxp3 expression in MLNC from *H. polygyrus* colonized animals has been detected earlier on the basis of quantitative PCR (56), no such effect of the infection was observed in this work. Comparing the protein expression level of Foxp3 within Tregs of naïve and infected animals based on flow cytometric analysis revealed no significant differences (data not shown). Another group recently also described an increase in regulatory T cell numbers during *H. polygyrus* infection (68). In accordance with the findings described in the present work, Finney et al. detected increased frequencies of $CD4^+CD25^+$ cells (reflecting the

increase of total $CD4^+$ cells) in MLN of *H. polygyrus* infected animals, with a delayed increase also seen in spleen. This was accompanied by an increased frequency of $CD103^+$ cells especially within the $Foxp3^+$ regulatory population, while the proportion of $Foxp3^+$ cells remained relatively constant (68).

Interestingly, similar phenotypic changes of Tregs are described for infections with *S. mansoni* (15). No overt changes concerning the ratios of Teff to Treg cells were detected in the inflamed liver. However, Baumgart et al detected an increase of $CD103^+$ Tregs, counterbalanced by a lower frequency of naïve-like Tregs, indicating a change in the phenotype of natural Tregs alongside with disease progression. Thereby the findings of changes within the regulatory T cell compartment presented in this work are in accordance with recent studies analyzing responses against the same pathogen and other helminths.

When carefully assessing the levels of Foxp3 expression within the subset of $CD4^+CD25^+CD103^+$ cells, only minor changes with more than 95% of these cells expressing Foxp3 were detected at each investigated time point. In contrast, the $CD4^+CD25^+CD103^-$ subset showed a significant decline in the Foxp3 expression rate in the acute phase of infection. This shows that the combined detection of CD25 and CD103 is very reliable for the detection of $CD4^+$ Tregs irrespective of the presence of recently activated, CD25 expressing Teff cells. Second, a significant proportion of Teff cells is detected according to CD25 expression during the acute phase of *H. polygyrus* infection. This is seen as a transient increase of $CD25^+CD103^-$ $Foxp3^-$ cells within the MLN. Interestingly, the vast majority of $CD4^+CD25^+$ cells express Foxp3 at later time points of infection. This might have several explanations, such as less recently activated T effectors being present within the lymph nodes due to migration to the site of infection, or fewer T cells being activated due to suppression by Tregs or reduced antigen presentation in the lymphatic organs.

While the percentage of $Foxp3^+$ Tregs stayed relatively stable in lymphatic organs during *H. polygrus* infection, a transient increase of Tregs was detected in the small intestine, which led to a significantly higher ratio of Tregs to conventional T cells at the inflamed site. The peak of Tregs in the small intestine at day 6 pi is in accordance with the time point of intense inflammation around tissue-invading larvae, characterized by accumulation of mainly granulocytes and, to a lesser extent, macrophages and $CD4^+$ T cells (7, 157). Of note, it has been shown

that Tregs not only control inflammatory responses driven by Teff cells, but also suppress innate immune responses in the absence of Teff cells (41). Hence it is conceivable, that Tregs accumulating at the site of infection might downregulate the activation of innate as well as adaptive immune cells.

The time point of the maximal Treg accumulation correlated with increased TGF-β1 and IL-10 levels within small intestinal tissue. There is increasing evidence for an essential role of TGF-β1 in inducing and expanding Tregs under certain conditions (35, 62). The coincidence of regulatory T cell accumulation and the peak of anti-inflammatory cytokine levels argue for Tregs as source for the latter. On the other hand, Tregs might be induced or expanded due to high TGF-β levels. It is conceivable that an increased level of this cytokine in the anyhow TGF-β rich milieu of the intestinal epithelium might favour such consequences. Furthermore, expanded or induced Tregs may upregulate CD103 expression under such conditions (31, 188). The increased frequencies of CD103 expressing Tregs found strikingly at the same time in draining lymph nodes (but not in spleen) might reflect cells returning from the intestinal site. Later on, increased amounts of parasite antigen recognized by expanded Tregs might explain the systemic increase of effector/memory-like Tregs detected in spleen. However, systemically upregulated TGF-β levels during *H. polygyrus* infection might also favour CD103 expression on Tregs irrespective of their antigen specificity. Interestingly, oral tolerance induced in MLN is described to spill over to a systemic unresponsiveness as detectable by antigen-specific Tregs in spleen. Finally, recent reports showed an increased production of IL-10 as well as TGF-β1 by lamina propria T cells after *H. polygyrus* infection (99, 201). These cells were referred to as regulatoty T cells due to the production of anti-inflammatory cytokines, however no data on expression of Foxp3 by these cells are available.

A role for gut-derived DC presenting parasite antigens in the draining lymph nodes also has to be considered. DC from the lamina propria (lp) have been shown to induce *de novo* Foxp3 expression in a TGF-β1 and retinoic acid dependent manner (219). So-called “alternatively activated” DC generated in presence of IL-10 and TGF-β express elevated levels of IL-10 and propagate functional $Foxp3^+$ cells from naïve T cell precursors (120). LpDC have been shown to hold a quiescent, tolerogenic state and to constitutively express IL-10. This DC status is probably favoured by TGF-β in the gut mucosa under homeostatic conditions and leads to

tolerization of naïve $CD4^+$ T cells after DC migration to the MLN (39). Others have shown that such mucosal DC (also expressing CD103), clearly differ from $CD103^-$ conventional DC in their mode of action. Probably $CD103^+$ DC are altered during their journey through the immunosuppressive intestinal environment. They produce or activate TGF-β and thereby support existing Tregs and induce $Foxp3^+$ cells from naïve T cells. The induction of $Foxp3^+$ cells has been shown to occur within days after antigen feeding (41). Finally, it is conceivable that Treg derived IL-10 and or TGF-β act back on DC to keep them in an inactive state, favouring tolerogenic conditions (136). Consequently, an increased anti-inflammatory cytokine production by DC at the site of infection and in draining lymph nodes might favour Treg expansion.

Taking these aspects of mucosal immunoregulation into account, the finding of increased intestinal TGF-β1 and IL-10 levels early after infection with *H. polygyrus* may be interpreted in several ways. First, the correlation between cytokine levels and increased frequency of Foxp3 expressing cells may argue for these cells as cytokine source. This may reflect an induced reaction favourable for the parasite development. Accumulated Tregs may also secrete TGF-β1 and/or IL-10 irrespective of antigen-specificity in response to the developing larvae leading to tissue damage. However, data on cytokine production of Tregs from MLN and spleen at day 6 pi show that $CD103^+$ Tregs secrete IL-10 specifically in response to parasite antigen already at this early time point (data not shown). Another explanation might be increased TGF-β1 production by epithelial and accessory cells in order to enhance tissue repair mechanisms (16). DC, macrophages or epithelial cells may contribute to the measured increase and be critically involved in expanding or inducing Tregs. Tolerogenic gut-derived DC may further support Treg maintenance in the draining lymph nodes, or effector/memory-like Tregs induced or expanded in the gut may migrate back to lymphoid compartments to interfere with the activation of Teff cells.

It is tempting to speculate whether the parasite exploits mechanisms of oral tolerance induction for its own advantage. The herein described kinetic and phenotypic changes of Tregs in response to *H. polygyrus* infection share similarities with aspects of this field of regulation. This might also explain, why certain compounds secreted by the parasite, especially during the acute intraepithelial phase of infection, fail to stimulate T cell proliferation and are poorly recognized by antibodies. As such secreted products are thought to have an immunomodula-

tory role, it might be of further advantage for the parasite if these molecules are tolerized by the host.

Although the data described in this study do not answer the question, whether infection with *H. polygyrus* induces antigen specific Tregs or expands the preexisting cell pool, it is conceivable that Tregs induced by the nematodes are recruited to modulate innate and adaptive immune responses. The resulting immunosuppression could control excessive pathology and favour prolonged parasite survival. Helminth-induced Tregs have been shown to be involved in immunomodulation in various helminth infections such as human infections with *Onchocerca volvulus* (194), or murine infections with *Litomosoides sigmodontis* (228), *H. polygyrus* (68) and *Schistosoma mansoni* (15, 122, 146). The general notion is that during helminth infections, Tregs might have the function to facilitate parasite survival and diminish immunopathology (138, 139), and the data presented in this work support this concept.

4.2 Tregs as source for IL-10 during infection: Treg specificity for the parasite

The role of IL-10 in suppressing protective immunity and immunopathology has been shown in various infection models. Doetze et al. detected IL-10 producing adaptive regulatory T cells (Tr1) specific for antigens of *Onchocerca volvulus* in infected patients (51). A mouse model for filarial infections revealed that IL-10 suppresses Th1 and Th2 responses, leading to prolonged parasitaemia (209). Another group showed for the same model of filarial infection that after depleting cells expressing CD25 (thereby mainly of regulatory phenotype), Th2 responses, including the production of IL-10, could be markedly enhanced (228). For infection with the intestinal nematode *T. muris* it was shown that animals deficient in IL-10 are highly susceptible to infection and display marked morbidity and mortality (197). To date, relatively few data on antigen-specific Foxp3 expressing cells in infections are available (15, 225, 216) and it is still under debate whether Tregs in nematode infections mediate suppression via soluble mediators such as IL-10 or TGF-β (19, 111, 150, 225). Increases in TGF-β1 expression by $CD4^+$ and $CD4^-$ cells and surface bound TGF-β1 on $CD4^+$ cells as well as increased plasma levels of active TGF-β1 during infection with *H. polygyrus* have been shown by others (68, 214, 252).

The data presented here suggest effector/memory-like Tregs from the chronic stage of infection as a main source for IL-10 produced in a parasite-specific manner, while only low levels of T cell derived active TGF-β1 were detected. Nakamura et al. revealed that $CD25^+$ Tregs secrete high amounts of TGF-β1 only under "maximal" stimulatory conditions, as seen *in vitro* in response to plate-bound αCD3-antibodies plus stimulation via the IL-2R, CD28 and CTLA-4, reflecting conditions of intense inflammation. In contrast, an ordinary level of stimulation by APC may lead to increased surface expression of TGF-β1, but not to augmented secretion (159). This might explain the low TGF-β1 levels in the *in vitro* assays described in this work. Interestingly, the production of active TGF-β1 by Teff cells was substantially higher than that by Tregs. However, it is conceivable that IL-13 produced by effector T cells may lead to the higher TGF-β1 levels detected in cultures of Teff cells, as it has been described earlier that IL-13 may induce and activate TGF-β1 *in vivo* (123). However, it is not known, whether this effect also occurs *in vitro*. Another group analyzing regulatory T cells during *H. polygyrus* infection detected increased levels of surface-bound TGF-β1 on conventional $Foxp3^-$ T cells, but not on the Treg subset of chronically infected mice (68). This does not necessarily reflect an increased expression of TGF-β1 by Teff themselves, as surface TGF-β1 might also originate from other sources and bind to TGF-β receptors such as TGF-β-RIII (159). By analyzing tissue samples from the small intestine, elevated TGF-β1 production at the site of infection was found at the early phase of infection, correlating with increased Treg numbers as discussed above.

The finding of an increased proportion of $Foxp3^+$ cells expressing an effector/memory-like phenotype raised the question, whether these cells specifically recognize the parasite and react with IL-10 production. Analyzing $CD4^+CD25^+CD103^+$ cells from spleen and MLN after restimulation *in vitro* revealed that they produce IL-10 in response to parasite antigen already at a very early time point of infection (6dpi). However, at this early time point, PCR data clearly show that a much stronger IL-10 signal originates from $CD4^+CD25^+CD103^-$ cells. As these contain a relatively high proportion of recently activated effector cells at this time (as seen by a decline in Foxp3 expression and a strong PCR signal for IL-4), it is conceivable that Th2 cells contribute to the high IL-10 levels at this stage of infection (this work, 68). This finding clearly differs from the pattern detected in the chronic phase of infection. Here, espe-

cially the effector/memory like Tregs produced high amounts of IL-10 in response to *H. polygyrus* Ag, which was augmented further when providing increased co-stimulation and nous IL-2. Clearly, an antigen-specific IL-10 response was not seen for naïve-like natural $CD4^+CD25^+CD103^-$ Tregs (possibly including a low contamination of recently activated effector cells). This leads to the conclusion that effector/memory-like Tregs show specificity for parasite antigens and that they represent the prominent IL-10 T cell source during the chronic phase of infection. Thus they might tone down pathogen-specific effector reactions *in vivo*. The fact that both Treg subsets produce high amounts of IL-10 when providing exogenous IL-2 can be explained by the inherent IL-2 exploitation mechanism of Tregs, described to prime these for IL-10 production (46).

While the general notion is that Th2 cells induced in response to *H. polygyrus* infection do not produce relevant amounts of IL-10 (74), a study also examining changes concerning Tregs in response to this pathogen showed high levels of IL-10 in MLNC cultures stimulated with parasite antigen and an increase in IL-10 producing $CD4^+$ cells during the chronic phase of infection (68). The present work also shows high levels of secreted IL-10 by MLNC/splenocyte cultures, especially during the acute phase of infection. While showing that probably, Teff as well as Treg cells contribute to this response in the early acute phase, the present data imply that especially effector/memory like Tregs release IL-10 in response to stimulation with the nematode antigen during the chronic phase of infection. In contrast, the data on intracellular cytokine detection in $CD154^+$ expressing antigen specific Teff cells, as well as DC cocultures, clearly show that Teff cells contributed marginally to IL-10 production, even when stimulated with Ag in the virtual absence of Tregs in DC cocultures. This implies that the lack of IL-10 production by Teff cells is not a consequence of suppression by regulatory T cells. This view is supported by the fact that Teff cells set free from the direct suppressive effect of Tregs *in vivo* restart to produce their normal cytokine pattern (113). This has been confirmed for infection with the filaria *Litosomoides sigmodontis*, where removal of cells expressing CD25 greatly enhances Th2 cytokine responses to parasite antigen, and, surprisingly, also the production of IL-10 *ex vivo*. However, it was not addressed, which cells are the source of this enhanced IL-10 production, and it has to be taken into account that the depletion via αCD25 antibodies does not completely remove regulatory T cells (19, 115, 245).

Infections with the protozoa *Leishmania major* and the cestode *Schistosoma mansoni* are the best analyzed parasite models with respect to the role of Tregs. A multitude of studies has shown the importance of IL-10 in controlling immunopathology as well as facilitating parasite survival. However, the described cellular sources of IL-10 differ depending on the pathogen and results may even differ in studies investigating the same model organisms. A compelling example for this is the infection with *Leishmania* species. Several studies showed that Tregs are a main source of IL-10 during *Leishmania* infections (211, 216) and that a strong IL-10 response in mice predicts failure of vaccination (216). Depletion of Tregs during infection somewhat mirrored the increased resistance seen in IL10 knock-out mice as well as after blocking the IL-10R *in vivo* (5). In clear contrast, recent studies implicated that Th1 or Th2 cells, rather than Tregs, are a prominent source of IL-10 in infections (5, 104). Human studies also differ with respect to the role and cellular source of IL-10 in their findings: Patients infected with *Leishmania donovani* (causing visceral leishmaniosis) showed that T_{reg} cells are not the main source of IL-10 in the spleen, nor did their depletion restore downmodulated IFN-γ responses against the parasite, while $CD4^+CD25^-Foxp3^-$ cells produced IL-10 after Treg depletion (164). Another trial analyzing the immune response against *Leishmania viannia braziliensis* claims CD25 expressing cells as the main source of IL-10 in cutaneous leishmaniasis (30). These studies clearly differ with respect to the *Leishmania* species, sources of analyzed T cells, the stimulation protocol and the methodology of IL-10 detection. Not all studies coanalyzed the expression of Foxp3 as a definitive Treg marker, rendering it more difficult to distinguish between natural Tregs, induced Tr1 cells or Th1 cells as sources for IL-10.

Studies investigating the immune response to *Schistosoma mansoni* showed that both innate and adaptive immune cells contribute to IL-10 production in response to the parasite. $CD4^+CD25^+$ T cells derived from liver granulomas developing around parasite eggs produced higher levels of IL-10 than their $CD25^-$ counterparts. Immunodeficient animals reconstituted with $CD4^+$ cells depleted of $CD25^+$ positive Tregs showed increased liver injury, pointing out that Tregs control immunopatholgy. In addition, also IL-10 derived from non-T cells had a protective effect (92). In a subsequent study, the presence of $Foxp3^+$ Tregs in granuloma was shown. However, neither $Foxp3^+$ Tregs from liver granuloma nor MLN or spleen did significantly contribute to IL-10 expression in this model, while Th2 cells are a dominant source for

IL-10. Still, the production of IL-10 by a fraction of Foxp3$^+$ cells could be shown after polyclonal restimulation. Of note, the cells were stimulated circumventing the TCR and co-stimulation by Ag-presenting APC, which might not mirror the finding of an antigen-specific cytokine response and may not represent the optimal form of stimulation for Tregs. The authors claim that Foxp3$^+$ Tregs suppress effector cytokine production independent of IL-10 in this model infection (15).

A clear hint for the specificity of Foxp3$^+$ Tregs for parasite antigen comes from a study investigating the role of Tregs in modulating responses to the eggs of *S. mansoni*. A substantial proportion of Foxp3$^+$ cells derived from the lymph node draining the site of egg application was found to produce IL-10 when cultured with DC pulsed with egg antigens (conditions similar to that used in the present work), showing the antigen specificity of these cells. Depletion of CD25$^+$ cells from egg-injected animals provoked an increase in Th1 as well as Th2 cytokine responses to egg antigen. However, this effect was shown to be independent of IL-10 (225). Interestingly, both CD25$^+$ and CD25$^-$ CD4$^+$ T cells were described to produce IL-10 in response to schistosome egg antigens, resulting in suppressed production of IL-12 by DC and thereby favouring the Th2 shift of the T cell response. Consequently, Tregs also take part in the Th2 bias seen during certain helminth infections (225).

Taken together, Foxp3$^+$ cells do not appear to be a main T cell source of IL-10 during schistosomiasis, while the data presented in this work strongly suggest effector/memory-like Tregs as a source for IL-10 in the chronic *H. polygrus* infection.

Although a contribution of Teff cells to the IL-10 production measured in DC culture assays cannot be excluded, the superior number of approx. 90% of Foxp3$^+$ Tregs imply these cells as a the source for IL-10. One may argue, that the isolation depending on expression of CD25 and CD103 may lead to a contamination by CD103 single positive cells, which were indeed present in ratios of 4-6% in all these assays. Given the fact that a relatively high proportion of these cells showed antigen-specificity according to expression of CD154 after restimulation, the risk of these Foxp3$^-$ cells as source for IL-10 may appear obvious. Still, no relevant IL-10 signal was detected within the CD154$^+$ antigen-specific CD4$^+$ T cell fraction, arguing against the notion of such non-regulatory CD103$^+$ cells as the source for the high IL-10 levels in these

assays. Second, roughly half of the $CD4^+CD25^-CD103^+$ T cells also express Foxp3, thereby again reducing the number of contaminating effector cells.

The analysis of changes concerning the lymphocyte composition of MLN after *H. polygyrus* infection presented in this work also included B cells. A large increase in B cell numbers was detected in MLN of infected animals and further work investigating these cells as possible source of IL-10 during *H. polygyrus* infection is needed, especially as B cells have been shown to produce IL-10 in response to pathogen derived antigens during protozoan and helminth infections (2, 78, 165, 173). B-1 cell knock-out mice were shown to display a higher susceptibility to filarial infection and a diminished IL-10 response to parasite antigen (2). However, the contribution of B cells to the cytokine response against intestinal nematodes has yet attracted low attention and to date few data on B cells as source of IL-10 in such infections are available. Work by Harris et al. showed that MLNs from mice infected with *H. polygyrus* have elevated numbers of effector B (Be2) cells displaying a Th2-like cytokine profile, but provide no data on IL-10 production by these cells in response to worm antigen (87).

The focus of this work was clearly on $CD4^+$ T cells, showing that especially effector/memory-like Tregs from mice infected with *H. polygyrus* produce IL-10 in an antigen-specific manner. The fact that effector/memory Tregs increase during infection with *H. polygyrus*, while the total proportion of Foxp3 expressing cells remains unchanged, argues for these activated Tregs to be directly addressed or induced by the nematode.

4.3 Suppressive activity of Tregs from *H. polygyrus*-infected animals

To test the hypothesis that infection with *H. polygyrus* not only quantitatively, but also qualitatively changes the phenotype of Tregs, classical suppression assays were performed with naïve-like and effector/memory-like natural Tregs. These *in vitro* assays demonstrated that $CD4^+CD25^+CD103^+$ Tregs were most potent in suppressing the proliferation of T cells when isolated from the chronic phase of infection. This peculiar suppressive capacity was seen when Tregs were present in physiological ratios (1:20), while infection derived effector/memory-like Tregs were generally more potent than their counterparts from naïve donors. The markedly stronger suppression by Tregs derived from infected animals did not depend on the presence of parasite extracts. Hence, the higher suppressive efficiency of

effector/memory-like Tregs from the chronic phase of infection does not require engagement of the Treg TCR by cognate parasite antigens or components addressing other receptors for the recognition of pathogens.

A particularly potent suppressive capability of $CD4^+CD25^+CD103^+$ Tregs in comparison to $CD4^+CD25^+CD103^-$ Tregs *in vitro* and *in vivo* has also been shown by others (124, 216). Most studies on the Treg-mediated suppression of T cell activation and proliferation *in vitro* point out that this mechanism is independent of soluble mediators (204). Lehmann et al. have shown, that CD103 expressing Tregs produce IL-10 in response to polyclonal activation, however, blocking IL-10 and TGF-β signalling did not restore the proliferative response of T cells (124). Still, one cannot rule out the contribution of IL-10 to the stronger suppression by effector/memory-like Tregs described in this work.

To investigate whether IL-10 was essential, suppression assays were performed in presence of antibodies blocking the IL-10R. Blocking IL-10 signalling did not restore the proliferative response of T cells, arguing for the irrelevance of IL-10 for the suppression measured *in vitro*. Another study with focus on regulatory T cells in *H. polygyrus* infection also pointed out that Tregs from infected animals display higher suppressive activity than their counterparts from naïve animals (68). Besides an increased IL-10 production in MLN $CD4^+$ cells, the authors found higher intracellular levels of TGF-β. Of note, both surface-bound TGF-β and CTLA-4 (a negative costimulatory molecule also thought to take part in cell-contact dependent suppression by Tregs) were detected to slightly increase on Teff cells, but not on Tregs from infected animals in the chronic phase (68). Hence, these molecules known to mediate suppression by Tregs under certain conditions *in vivo* and *in vitro* (242) could not be linked directly to the higher efficiency of Tregs from chronically infected animals (68). However, the increased expression of CTLA-4 on the surface of conventional T cells might contribute to mutual downregulation of effector responses irrespective of the action of Tregs. The reduction of free tryptophane levels due to enhanced expression of indoleamine 2,3-dioxygenase by APC could be another suppressive mechanism not necessarily involving Tregs (242). Furthermore, it may be conceivable that T cells expressing CTLA-4 may be converted to Foxp3 expressing cells or inducible TGF-β secreting T cells by engagement of the negative costimulatory receptor by APC, as described recently (127).

While probably not contributing to an elevated suppressive capacity of Tregs during *H. polygyrus* infection, it has been shown for human filaria infections that the strongest increase in CTLA-4 expression occurs on $CD4^+CD25^+$ cells, and that blocking of CTLA-4 increases the Th2 response while decreasing the Th1 response (210). Taylor et al. showed for a filarial infection model that depletion of $CD25^+$ cells alone did not restore the parasite specific response of $CD4^+$ cells, while enhancing the killing of adult parasites when performed in combination with neutralization of CTLA-4 (226). A recent study investigating the role of $CD25^+$ for human filaria infections and CTLA-4 in schistosome infections showed that $CD25^+Foxp3^+$ as well as conventional T cells upregulate CTLA-4 during infection. Interestingly, the application of anti-CTLA-4 antibodies led to dramatic weight loss, accompanied by elevated eosinophil and Th2 cells numbers in liver granuloma and increased IL-4 production by splenocytes, while no such effects were seen when starting the treatment in the chronic phase of infection. CTLA-4 is probably needed to control the strong initial Th2 response to the parasite, otherwise leading to excessive Th2 dependent inflammation, while during the later chronic phase, other CTLA-4 independent regulatory circuits may have developed (245). Evidence for this is provided by a study investigating the effect of CTLA-4 blockade on tolerance to allergens. While tolerance induction is altered during the priming phase, CTLA-4 blockade had no effect once tolerance had been established (3). Consequently, the role of CTLA-4 with respect to Treg cell-mediated or -independent regulation may differ depending on the pathogen and the state of infection. Interestingly, studies analyzing the role of CTLA-4 during infection with the intestinal nematode *N. brasiliensis* revealed that blockade of CTLA-4 signalling accelerates and enhances the protective Th2 immune response and leads to decreased fecundity and worm burdens (143). Still, as Tregs in the chronic phase of a *H. polygyrus* infection do not show increased surface expression of CTLA-4, it seems rather unlikely that CTLA-4 is directly involved in the increased suppressive efficiency of infection derived Tregs. While clearly showing that the effector/memory-like subset of Tregs derived from *H. polygrus* infected mice displays an increasing suppressive activity along the infection, this work is unable to answer the question, which mechanism may lead to this phenomenon. It is clear from *in vivo* studies that the homing properties of $CD103^+$ Tregs enable them to control inflammatory responses directly at the site of infection more efficient than naïve Tregs (97), but the *in vitro* mechanism of this higher efficiency is not yet resolved.

Having ascribed an extraordinary level of activity to infection-derived Tregs cells, a further step was to analyze their role *in vivo* by performing adoptive transfers.

4.4 The role of Teff and Tregs *in vivo*: Adoptive transfer studies

4.4.1 Transferred Tregs do not alter worm burden

A multitude of studies have analyzed the role of Tregs during parasitic infections, mostly by inactivating cells expressing CD25 or using immunodeficient animals (see appendix). The depletion method has the disadvantage that not only Tregs are addressed, but also recently activated Teff cells expressing the IL-2 receptor α-chain. Recently, the question arose whether the antibody generally applied leads to downregulation of CD25 and not to depletion of regulatory T cells, seen as minor changes in the level of $Foxp3^+$ cells despite significant reduction in $CD25^+$ cells (245). Indeed, the application of αCD25 antibodies may rather functionally inactivate Tregs instead of removing them, which is also depending on the applied antibody clone (115).

The model chosen in this work was based on adoptive transfer of T cells from *H. polygyrus* infected donors into immunocompetent recipients, which were infected with *H. polygyrus* according to different protocols. This should scrutinize the hypothesis, whether transfer of Tregs might interfere with the primary Th2 response against *H. polygyrus*, entailing increased worm burdens. $CD4^+$ T cells have been described as essential for protective immunity to reinfection with *H. polygyrus* (7, 236, 237). A parasite-beneficial effect of a Treg transfer appeared conceivable, as especially $CD103^+$ effector/memory-like Tregs displayed a high suppressive activity on $CD4^+$ T cell proliferation in *in vitro* assays. Furthermore, studies on protective immunity against *H. polygyrus* have shown that severe combined immunodeficient (SCID) mice lacking T and B cells and mice depleted of $CD4^+$ T cells harbour higher worm burdens than wildtype or untreated controls (238, 239). In addition, the BALB/c mouse strain chosen for analysis in this work is a strain favouring strong Th2 responses against GI nematodes as well as other parasitic infections. These mice show an intermediate susceptibility for *H. polygyrus* infection. This is seen in the chronic phase with generally lower numbers of adult worms persisting for prolonged periods of time as compared to Th1/Th2 mixed

type response strains, such as C57/BL6 mice (own observations). Hence, it seemed conceivable that a higher number of worms would persist during the chronic phase of infection after transfer of pathogen-experienced Tregs.

However, in spite of the high suppressive capacity of $CD103^+$ Tregs *in vitro*, the *in vivo* transfer model did not reveal an influence of Treg enrichment on worm burden. Transfer of $CD4^+CD25^+CD103^+$ as well as $CD4^+CD25^+CD103^-$ Tregs did not alter worm numbers. Maybe more strikingly, the transfer of total $CD4^+CD103^+$ cells led to decreased worm burdens. This outcome of the transfer of a mixed population, consisting predominantly of Tregs and few antigen-specific Teff cells, argues strongly for an insufficiency of Tregs to suppress the protective effector response induced by antigen-specific Teff cells *in vivo*. The discrepancy between the suppressive activity of $CD103^+$ Tregs *in vitro* and the failure to interfere with worm expulsion *in vivo* might be due to multiple reasons: First, Tregs might be involved in suppressing pathology, but not in facilitating worm survival, as described for infection with another GI nematode (19) and fluke worm infections (92, 122). Second, the conditions *in vitro* versus *in vivo* were clearly different. *In vitro*, Tregs drastically inhibited the proliferation of naïve $CD4^+$ responder T cells after polyclonal stimulation, whereas *in vivo*, Tregs had to combat antigen-specifically activated Teff cells. Since it has been shown recently that Tregs share the expression of homing receptors with T effector cells and thereby co-locate in different microenvironments (205, 207), it is rather unlikely that Tregs failed to suppress co-transferred effector cells due to differential homing of both subsets. Finally, Tregs might not survive the transfer regimen.

Recently, Tregs have been shown to proliferate vigorously in presence of DC infected with *Leishmania major*, while Tregs purified from lymph nodes draining the site of infection showed a two-fold increase in annexin V labelling (216), arguing for these cells to be highly susceptible for apoptosis. The authors also show that after removal of the parasite, Tregs purified from the regional lymph nodes fail to proliferate in response to infected DC. Thus, antigen-specific Tregs seem to be rapidly eliminated in the absence of their cognate antigens. This may imply that in the transfer model presented in this work, highly activated Tregs derived from chronically infected donors might succumb to induced cell death as not being directly addressed by APC presenting their cognate antigens. Therefore, in some experiments,

mice in the early phase of infection (6 dpi) were used as recipients for adoptive transfers, assuring that APC presenting the parasite antigens were present. However, no significant differences between both regimens (transfer prior to infection vs. infection prior to transfer) were detectable.

A more direct approach to verify the survival of transferred cells was performed using high numbers of GFP-expressing total $CD4^+$ cells from chronically infected donors. Assessing the expression of CD103 and CD25 on the surface of GFP expressing cells revealed slightly lower proportions of $CD25^+CD103^-$ and $CD25^+CD103^+$ cells compared to endogenous $CD4^+$ cells. However, $Foxp3^+$ cells were detectable among GFP^+CD4^+ cells, albeit at lower levels than observed in the corresponding endogenous cell subsets. This analysis was performed comparing animals with a present infection to those with subsequent infection after transfer. No differences were observed with respect of GFP^+ proportions or the levels of Tregs within the transferred population. Reanalysis of recipients 3.5 weeks post transfer and infection revealed similar proportions of GFP^+ cells, however, virtually no cells expressing CD25 or CD103 were detectable (with exception of CD103 expressed on most transferred cells in the intraepithelial intestinal compartment), arguing for the depletion of transferred Tregs after a prolonged time period.

Taken together, these data show, that a certain proportion of transferred T cells survived for several weeks, although transferred Tregs were only detected within the first week after transfer. The CD154-technique might give a hint whether GFP^+ expressing $CD4^+$ T cells still detectable after several weeks are predominantly antigen-specific effectors or not related to infection.

Interestingly, it has been shown in vitro, that IL-4 may render Teff cells refractory to suppression by Treg cells, while favouring proliferation of the latter (171). To date, it is not clear whether such a mechanism might account for Th2 effector cells escaping a tight control by Treg cell also *in vivo*. However, the high IL-4 levels provoked early after infection with *H. polygyrus* might contribute to maintenance and expansion of Tregs as a feedback loop to control excessive inflammation. On the other hand, Teff cells insensible to suppression by Tregs due to IL-4 production might explain the lack of control of protective effector responses against secondary infections. This might also explain the finding of protection after transfer

of effector/memory-like Tregs in combination with effector cells. Even in case of co-localization of transferred Teff and Treg cells at the site of infection, Teff cells might escape suppression by an accelerated IL-4 production. Finally, this model could also contribute to the understanding of the downmodulated parasite-specific effector responses in the chronic phase of infection. While the strong IL-4 response during the acute phase might also favour Treg outgrowth, the increasing amounts of deactivating cytokines such as IL-10 produced by expanded Tregs may act back on Teff cells as well as APC and thereby gradually counterregulate the effector response, favouring prolonged parasite persistence.

While the transfer of infection-derived T cells with regulatory capacity clearly showed effects on inflammatory disease models such as colitis or asthma (117, 141, 252), less studies investigated the impact of such transfers on responses towards the priming pathogen (see appendix). A model analyzed in detail is the infection with the protozoan *Leishmania major*. Transfer of primed $CD4^+CD25^-$ T cells to immunodeficient recipients rendered them highly resistant against a challenge infection. In contrast, recipients of $CD4^+CD25^+$ cells did not control parasitaemia. Importantly, transfer of $CD4^+CD25^+$ cells to immunocompetent mice led to preferential accumulation of Tregs at the site of infection and rendered recipients highly susceptible to infection with a non-healing phenotype (23). Further studies showed that transferred infection-derived Tregs or parasite-specific $Foxp3^+$ cell lines led to disease reactivation in mice that had efficiently controlled a primary infection (149, 216). Of note, in another study using the same mouse strain, but another *Leishmania* species (*L. amazonensis*), the transfer of $CD25^+$ cells had opposing effects, seen in decreased parasite loads in recipients (106).

Studies on immunity against microbial infections also revealed effects of transferred $CD25^+$ Treg cells. *In vitro* stimulated $CD4^+CD25^+$ cells transferred to immunocompetent mice improved bacterial clearance and protection in a sepsis model (93). Efficient clearance of *Mycobacterium tuberculosis* infection in an immunodeficient (ID) mouse model was only seen when naïve $CD4^+$ T cells were transferred after depletion of $CD25^+$ Tregs (118). Finally, naïve Tregs were shown to inhibit intestinal inflammation in response to *Helicobacter hepaticus*, although not influencing the bacterial load in an ID mouse model (141).

Transfer of $CD4^+$ T cells to ID animals infected with the fluke worm *S. mansoni* revealed that recipients suffer from increased morbidity and mortality, when CD25 expressing cells are

depleted before transfer (92). However, data on the influence of transfers of complete or CD25-depleted $CD4^+$ T cells on the parasite itself are lacking. A study investigating the involvement of Toll-like receptor 2 (TLR-2) in Treg responses during *S. mansoni* infection showed that functional inactivation of Tregs by αCD25 antibodies enhances egg destruction. TLR-2 knock-out animals failed to expand Tregs in response to the parasite, while displaying enhanced T helper cell responses, pathology and decreased egg output. This was reversible either by transfer of $CD25^+$ T cells from immunized wildtype donors or Tregs primed *in vivo* by schistosome egg antigen (122). However, it is clear that the effect on viable egg burdens in schistosome-infected animals does not necessarily reflect an effect on adult worms.

Taken together, few studies have analyzed the impact of regulatory T cell on immunity against pathogens, especially helminths. No data are available on changes concerning parasite fitness or cytokine response against *H. polygyrus* after Treg manipulation either by functional inactivation or transfer. A study investigating the impact of the regulatory cytokine response and functional inactivation of Tregs during infection with the GI nematode *T. spiralis* was able to show that inactivated Tregs do not result in changes of larval burdens (19). However, this infection displays special features, such as a very short-lived intestinal adult stage and a chronic phase caused by dormant larvae present in the skeletal muscles. Therefore the present work represents the first study on manipulation of the regulatory T cell circuits in response to a chronic GI nematode infection. Although no direct effect of transfer of Tregs on parasite survival was detectable, future studies will show whether interfering with the priming of regulatory T cells during infection by depletion of $Foxp3^+$ cells and/or inactivation via αCD25 antibodies may influence parasite development.

4.4.2 Transfer of protection by infection-derived $CD4^+$ cells

This works shows that $CD4+CD25^-CD103^-$ Teff cells transferred a partial protection against an intestinal nematode infection to naïve or recently infected recipients. The effect could be achieved by the surprisingly small number of half a million cells, while transferring higher numbers did not lead to further reduction of parasite burden (data not shown). Adoptive transfer of naïve $CD4^+$ or infection-derived $CD4^-$ cells in recipients that were subsequently infected with *H. polygyrus* did not show an effect on worm burden (data not shown). This is the first description of $CD4^+$ T cells derived from an ongoing *H. polygyrus* infection to mediate

protection after transfer, while one earlier study revealed protection against the GI nematode *T. muris* by transfer of infection-derived T cells (123). A recent study analyzing the development of central and effector memory T cells after infection with *T. muris* could show that transfer of memory cells renders naïve recipients resistant to infection (258). Anthony et al. recently also showed protection against *H. polygyrus* infection after transfer of MLN cells from drug-cured mice (7).

As determination of fecal egg counts in the present work showed no consistent pattern, it is not possible to clearly point out the developmental stage of the parasite addressed by presumably increased effector mechanisms. In most transfers, the egg output of $CD4^+$ T cell recipients resembled that of control groups during the first three weeks of infection, arguing for a similar proportion of parasites successfully developing to adults. In other experiments, the egg output of $CD4^+$ recipients was decreased from the beginning, arguing for the larval stage as target for protective effector mechanisms.

The definite protective immune reactions to secondary infections with *H. polygyrus*, which one might suppose to resemble the mechanisms driven by transfer of adaptive immune cells from parasite experienced donors, are not fully elucidated yet. A recent study re-evaluated the T cell dependence of immunity against challenge infections with *H. polygyrus* (7). The protective effect of transferred $CD4^+$ Th2 memory cells was more pronounced as compared to what is described in the present work for the transfer of Teff cells from an ongoing infection. Whether differences in e.g. the homing receptor repertoires or susceptibility to induced cell death between memory and effector T cells might be responsible for this phenomenon is not yet clear.

Anthony et al. showed, that depleting $CD4^+$ cells during a secondary *H. polygyrus*-infection abrogates protection, but only when performed before adult worms appear in the gut lumen. In line with this, unimpaired secondary immune reactions seem to target the larval stage, as few L4 were recovered from secondary infections (7). This clearly shows that the larval stage is addressed by protective mechanisms, and that these strictly depend on $CD4^+$ cells. The present work shows similar data concerning protection by Teff cell transfer shortly before or after a primary infection. As neither numbers of successfully developing larvae were calculated in this work, nor did the fecal egg output show sufficient consistency, the protective

mechanism of effector cells cannot be ascribed indubitably to target the parasite larvae. Still, the larvae may be efficiently addressed under both transfer/infection timeline regimens, as the larval stage was still present in experiments with transfer 6 days post infection.

The larval stage is vulnerable to damage by innate effector cells, namely alternatively activated macrophages (AAM) (6, 7). It is a target of an arginase-dependent effector mechanism by IL-4R expressing AAM, which accumulate around the developing larvae during the intraepithelial phase. These macrophages are probably activated by IL-4 and/or IL-13 producing Th2 cells. Both $CD4^+$ T cells and AAM appear in low numbers around the tissue invasive larvae during primary, but in high numbers after secondary infections with *H. polygyrus,* correlating strikingly with the finding of protection against challenge infections. Both cell types are critically involved in worm expulsion, as depletion of either $CD4^+$ cells or macrophages abrogates protection (7, 237, 238). This is the first definitive finding that AAM are involved in mediating protection against *H. polygyrus* (7). As depletion of macrophages almost completely abrogated the resistance to reinfection, it is very likely that these cells are rapidly induced during a memory response, probably as a mean of co-localized Th2 effector cells readily producing IL-4 and IL-13 (7). The finding of protection after application of memory T cells developing after a drug-cured primary infection also argues for these cells migrating to the site of infection, where they might rapidly provoke the alternate activation of macrophages combating the tissue dwelling larvae. This view is supported by the fact that such memory T cells express homing markers needed for the entry to inflamed sites (153).

However, preliminary experiments, where *in vitro* generated Th1 or Th2 cells were applied to primary or secondary infections with *H. polygyrus*, neither revealed an overt migratory behaviour of T cells directly to the intestine, nor to Peyer's patches or MLN (U. Hoffmann, personal communication). Given the fact that transfer of $CD4^+$ T cells from drug-cured (7) or ongoing primary infection (this work) leads to protection, the question arises, whether the protective circuits take part predominantly in the infected organ itself or to which extent cells immigrating from other compartments are needed. Furthermore, this work analyzed the migratory behaviour of cells in separate approaches, revealing no overt accumulation of transferred cells at the site of infection or draining lymph nodes. However, this would need more

in depth analysis, as it might be possible that the frequencies of antigen-specific cells differ between lymphoid or epithelial control sites and those afflicted by the infection.

Summing up, this work is one of the few studies showing a direct effect on parasite burden after transfer of effector cells from an ongoing primary infection. This contributes to the understanding of the scheme of immunity against GI nematodes, being highly dependent on Th2 $CD4^+$ T cells and, as described recently, on alternatively activated macrophages as effector cells. The protective effect shown here is in accordance with the concept that resistance to gastrointestinal helminths is dependent on $CD4^+$ Th2-type immune responses as shown in animal models with *H. polygyrus* or *N. brasiliensis* (60, 238, 239) as well as in humans infected with *Ascaris lumbricoides* and *Trichuris trichiura* (103, 235).

The data indicate that tipping the balance of Teff cells during infection strongly influences survival of parasitic nematodes, while Tregs may have functions that are not directly related to worm persistence. Although this work failed in ascribing a parasite beneficial effect to regulatory T cells by transfer studies, this does not necessarily mean, that these cells may not be specifically addressed by the parasite and favour its survival. Further work is needed to verify or revise the finding of "ineffective" Tregs with regard to host and parasite protection. Addressing this by the usage of the Treg-transgenic DEREG mouse model may permit new insights in the role of Treg cells, as these can be depleted specifically (119). Of note, this might not be restricted to the effect on parasitaemia, but may also contribute to the understanding of the control of immunopathology. The immunological backround of the transgenic mouse strain is of advantage, as C57BL strains show lower signs of inflammation due to developing larvae than the BALB/c strain used in this work (own observations). Manipulating Tregs thereby may provoke stronger signs of inflammation and could also have an influence on the alternate activation of innate effector cells.

4.5 Distribution of antigen-specific CD4+ T cells during *H. polygyrus*-infection

This work provides data on distribution and cytokine profile of parasite specific $CD4^+$ T cells using the marker CD154 (CD40-ligand), recently shown to exhibit exquisite specificity for antigen-activated cells in the human and mouse system (71, 110). Others have shown the reli-

ability of this method for the detection of antigen-specific T cells in a transgenic adoptive transfer model, where all cells reacted with expression of CD154 after restimulation with their cognate peptide antigen *in vitro* (110). Parts of the data presented in this work have been published recently in the aforementioned study and the analysis of effector responses applying the CD154 technique was performed with the help of D. Kirchhoff.

The *H. polygyrus* infection model revealed low percentages (<1%) of antigen-specific $CD4^+$ cells present in MLN and spleen according to CD154 expression in the chronic phase, producing predominantly IL-4 and IL-13. Albeit cytokine producing $CD4^+$ T cells were also detected in low percentages among $CD154^-$ cells, the responders to worm-antigens where highly enriched within the $CD154^+$ population, pointing out the high specificity of this method.

In contrast, much higher frequencies of IL-4 producing cells are described for infections with *H. polygyrus* in IL-4 reporter mice (153). Of note, Mohrs and colleagues provide data showing that not all GFP-expressing cells produce IL-4 in response to restimulation with parasite extracts, arguing for a marked contribution of bystander Th2 cells not directly related to the infection to the strong reporter signal. The authors also claim that $CD4^+$ T cells recently producing IL-4 *in vivo* were impaired in their capacity to produce cytokines in response to TCR-mediated stimulation *in vitro* (153). This finding might, in part, explain the lower frequencies of antigen-specific cytokine producers detected *in vitro* in this PhD work. It is also possible that relying on the levels of CD154 and cytokine expression leads to an underestimation of the frequencies of antigen-specific cells, as the method might not favour an optimal response of antigen-specific $CD4^+$ cells. Some imponderables possibly involved in suboptimal responses were excluded: As total spleen or MLN cells from the chronic phase of infection were used, the present APC might have been impaired in presenting parasite antigens to T cells. Hence, in some assays APC derived from naïve animals or bone marrow derived DC were used with $CD4^+$ T cells from chronically infected donors. However, this did not favour a stronger CD154 expression on T cells (data not shown). In addition, T cells from the acute phase of infection (10 dpi) showed only slightly increased levels of CD154 expression (data not shown), excluding downmodulatory effects in the chronic phase as explanation for the low responder frequencies detected. Finally, the CD154 technique was applied to MLNC cultures depleted of cells expressing CD25 and/or CD103. Clearly, this did not favour higher ex-

pression rates of CD154, although it has to be taken into concern that possibly recently activated cells might have been depleted *ex vivo* before culture (data not shown).

Despite the marked difference between the frequencies of antigen-specific $CD4^+$ T cells presented in this study compared to the IL-4 reporter system, this work shows that the detection of CD154 expression facilitates the analysis of a broader spectrum of pathogen-specific cytokine responses in a non-manipulated immune system. The combination of CD154 detection with the reporter system might more precisely define the frequencies of antigen-specific cytokine producers in response to *H. polygyrus* infection.

Interestingly, the work with GFP-IL-4-reporter mice showed the systemic dissemination of Th2 cells after a primary *H. polygyrus* infection and these studies introduced the peritoneal cavity as a major site for antigen-specific cells driven by *H. polygyrus* infection, indicating that non-lymphoid sites serve as reservoir of memory Th2 cells. Peritoneate exudates cells, besides MLNC and splenocytes, accounted for the highest absolute numbers of GFP^+CD4^+ cells during a primary *H. polygyrus* infection (153). These cells were shown to express the integrin $\alpha_4\beta_7$ in high densities, a molecule with key role in homing to the intestinal site (83). As this population did not appear to be recently activated or contribute to IL-4 production *in vivo* and displayed very low apoptotic potential, it is seen as a memory pool existing for prolonged periods of time (153). It would be interesting to investigate, whether GFP^+CD4^+ cells persisting within the intestinal wall of drug-cured mice are essential for mediating protection to secondary infections, or whether the pool of memory cells with the ability for an accelerated IL-4 response is indispensable, especially as these cells express the gut homing marker $\alpha_4\beta_7$.

In conclusion, applying the CD154 technique to the investigated GI infection model facilitated the analysis of parasite-specific cytokine responses. It revealed that specific effector cells are highly enriched within $CD103^+$ expressing cells, a finding with striking similarity to $CD103^+$ effector/memory-like Treg cells producing IL-10 in a parasite-specific manner. As regulatory $Foxp3^+$ cells do not upregulate CD154 in response to stimulation, the CD154 technique is not applicable to scan for parasite-specific regulatory T cells. However, it may help to investigate the cytokine production pattern of rare reactive cells in the chronic infection, where the reactivity of T cells is suppressed.

4.6 Treatment of colitis with filarial cystatin

Besides the investigation of the role of effector and regulatory T cells in a chronic intestinal nematode infection, a second aim of this work was to test an immunomodulatory nematode protein for its effect on an *in vivo* model of intestinal inflammation (colitis). Mice fed with the high molecular weight chemical dextran sodium sulfate (DSS) suffer from a colitis-like disease. This is characterized by the destruction of the colon epithelium, which in turn leads to increased contact to luminal contents and heavy inflammation. The reactions provoked by DSS treatment are similar to the findings in patients suffering from inflammatory bowel disease (IBD), especially the form of ulcerative colitis (UC) (54). The characteristic inflammatory response seen in UC patients by infiltration of neutrophils and macrophages, which release chemokines and cytokines, is also seen in the acute DSS colitis model. The chronic model with repeated DSS-feeding cycles resembles even closer to the UC situation, with exacerbated dysfunctional immune responses, mediated by activated Th1 or Th2 cells in the gut mucosa.

The elucidation of immunological changes by treatment with helminth products is of special interest, as this might provide a useful tool to interfere with or even disrupt the inflammatory cascade, yielding the possibility of precise, targeted therapies for IBD. A broad spectrum of studies conducted with helminth infections or application of worm eggs revealed the potential of these organisms to interfere with such inflammatory processes, which urgently demand for more satisfying treatment (249).

As an example, studies applying *H. polygyrus* to animals defective in IL-10 signaling inhibited a fully established colitis by interfering with Th1 cytokine production and increasing regulatory T cell numbers (56). Subsequent studies revealed a role for parasite-induced lamina propria $CD8^+$ regulatory T cells suppressing colitis independently of IL-10 and TGF-b in RAG/IL-$10^{-/-}$ mice (150) as well as IL-10-producing $CD4^+$ T cells in a trinitrobenzenesulfonic acid (TNBS) colitis model (201). Furthermore, the parasite-driven induction of TLR-4 expression on $CD4^+$ T cells, producing TGF-β in response to receptor triggering by LPS, was proposed as a central mechanism of interference with inflammatory responses (99). Interestingly, the same nematode also interferes with Th1 driven gastric inflammation induced by *Helicobacter pylori* (70).

Of note, also prior infection with the short living intestinal nematode *T. spiralis* (a parasite inducing massive pathological changes in the mouse intestine) was shown to ameliorate chemically induced colitis, even though the intestinal stage of the parasite was not present anymore when the chemical treatment was started (107). While it has been noticed for long, that suppressive macrophages develop in the IL-4/IL-13 rich milieu sustained by parasitic helminths (134, 169), only recently it has been shown that infection with schistosomes protects mice from colitis via a macrophage-mediated mechanism. Animals showed marked infiltrations of macrophages to the lamina propria and protection was abrogated by their depletion (208). These macrophages were not of the alternatively activated type, as described for cells infiltrating the intestine in response to *H. polygyrus* infection (7). Nevertheless one may speculate that interfering with the number and state of activation of macrophages in the intestine is a common (Treg-dependent or –independent) mechanism of helminth-driven modulation of intestinal inflammation.

Taken together, the propensity of helminth infections to entail a strong Th2 and regulatory cell response has been proposed to reduce the severity of experimental IBD. Human studies with IBD patients showed, that treatment with eggs of *T. suis* (followed by an abrogated infection due to the mismatching host/parasite combination) significantly ameliorates CD (211, 212).

Unlike CD, where the Th1 pathway is highly active and thought to significantly contribute to disease development, UC and also the DSS colitis model are seen as a Th1/Th2 mixed type response. Hence, the propensity of helminth infections to stimulate a Th2 shift may be essential in counterregulating an excessive Th1 response in CD patients or certain colitis models (249), but the mode of action probably also comprises other mechanisms such as activating regulatory cell and modulating strong Th2 responses. This becomes clear from studies showing a beneficial effect of worm infection also on defective Th2 type responses, like asthma (252, C. Schnöller et al. in preparation). Great interest has been paid to the question, what parasite structures might be critically involved in modulating such scenarios.

To address this, a defined filarial protein, the cystatin Av17, was tested for its effect on the DSS colitis model. Although derived from a nematode not associated to mucosal tissue, but residing subcutaneously and between skeletal muscles, the application of this protein led to

striking amelioration of intestinal inflammation in an acute model of colitis. This was seen as reduced epithelial damage, diminished size of cellular infiltrates and wide areas of healthy mucosal structure with plenty of goblet cells present. As the infiltrations observed in colitic mice are mainly composed by granulocytes and macrophages, and Av17 is known to exert its immunomodulatory effect on the latter, it was of interest to see whether depletion of macrophages might interfere with the ameliorating effect of the cystatin-treatment. Due to the problem of high animal losses during such experiments, this cannot be answered in this work. However, histopathological comparison of animals fed with DSS only and those co-treated with cystatin revealed no overt differences in intestinal macrophage numbers in animals receiving Av17. This supports the assumption, that macrophages, if essential for the Av17 effect in this model, are not simply affected in numbers, but in their phenotype.

It is clear from the experiences made with the DSS colitis model in this work that this model displays certain advantages for the investigation of the modulatory effect of parasite-derived components. The clearest advantage is that the model is not very time-consuming. The colitic reaction is rapidly induced in immunocompetent animals and the one-week treatment phase of the acute model may reveal hints on the efficiency of the tested component quickly. In addition, the model is easy to handle and relatively low amounts of the tested component may be applied (in case of filarial cystatin, 100µg per animal were needed to see a clear effect). On the other side, there are obvious disadvantages: Although methodologically easy to handle, the chemical treatment is a critical point. Albeit fed with the same charge of DSS, small variances in the dosage led to insufficient onset of inflammation or to uncontrollable reactions, seen in high losses of experimental animals. This became clear especially in the chronic model, where roughly half of the animal did not survive several treatment cycles. To avoid the situation of insufficient group sizes for statistical analysis, the animal number clearly has to be raised. Variances in length of recovery phases between treatment cycles make it complicated to compare data from independent experiments.

The described uncertainties rendered it extremely difficult to investigate the mechanisms of Av17-action in this model. As filarial cystatin has been described earlier to induce IL-10 by immune cells, especially macrophages (88), both immune components were addressed also in the colitis models to determine which effector mechanisms Av17 is leaning on. Ap-

proaches aiming to further define the role of macrophages as well as IL-10 failed due to high animal losses. Few mice survived the treatment with clodronate-filled liposomes (to deplete macrophages) or the application of αIL-10R antibodies with or without co-treatment by Av17. Tendencies of worsened inflammation in Av17 treated animals with reduced intestinal macrophage numbers and impaired IL-10 signaling cannot be conclusively interpreted due to few surviving animals also in DSS control groups.

Filarial cystatin applied in a model of airway hyperreactivity revealed a beneficial effect on development of asthmatic responses to the model allergen ovalbumin. Here, the effect could be ascribed in part to macrophages, IL-10 and regulatory T cells. Depletion of macrophages and functional inactivation of Treg by αCD25-antibodies as well as the application of antibodies directed against the IL-10 receptor partially reverted the downregulating effect of Av17 (Schnoeller et al.; JI 2008).

As described above, at this time no conclusion on the effector mechanism of Av17 can be drawn from the conducted colitis experiments. However, a model of spontaneous colon inflammation in IL-10 knockout mice points out that this cytokine is centrally involved to control intestinal inflammation (9). Thus, it may be conceivable that blocking IL-10 signaling in combination with the strong inflammation induced by chemical disruption of the intestinal barrier function is not the method of choice for the desired mechanistic approach. Even in case of a clearly reverted effect of Av17-treatment in combination with blocked IL-10 signaling, this would presumably be hard to distinguish from the exacerbating effect of the antibody-treatment alone. Similar difficulties have to be expected concerning the investigation of the role of macrophages. Data from a study on macrophage depletion in the DSS model clearly showed, that these cells are involved in suppression of colitis. Disparate from the IL-$10^{-/-}$ model, where depletion of macrophages inhibits intestinal inflammation (probably a consequence of the removal of constantly activated innate cells supporting inflammation), wild type animals react with a worsened disease development (180). This is probably due to the removal of the resting, relatively quiescent macrophages residing in the healthy intestinal tissue which favor anti-inflammatory responses. As macrophages emigrated recently from the blood have the potent ability to produce inflammatory cytokines (220), the replacement of depleted cells by emigrating macrophages may result in a strong pro-inflammatory re-

sponse. Of note, the depletion by clodronate liposomes in the present work was never complete and histological sections showed signs of quick replacement by emigrating macrophages. Furthermore, tissue residing macrophages (especially those in the IL-10 and TGF-β rich intestinal milieu) are known to contribute to tissue repair under inflammatory conditions (174). Thus, the removal of these cells may result in exacerbated colitis per se, in addition to confounding the beneficial effect of Av17 treatment.

Still, it is very tempting to speculate, that Av17 might address macrophages not only in the model of allergic airway hyperreactivity, but also in the colitis model. Resident macrophages seem to be prone to keep in check the balance between the defense against pathogens (by their unimpaired phagocytic capacity under homeostatic conditions) and tolerogenic features (by impaired pro-inflammatory cytokine responses and production of IL-10). Thus, it is conceivable that macrophages of a more pro-inflammatory type are attracted to the intestine in response to the severe tissue injury induced by DSS-treatment. This response (eventually in combination with activated regulatory T cells) might be the point of intervention of the immunoregulatory filarial cystatin. It might keep emigrated macrophages in a quiescent state, while furthermore supporting the anti-inflammatory state of resident cells.

As directive for future work, the identification of the modulatory mechanism of Av17 during intestinal inflammation would presumably be better addressed in alternate models. The SCID colitis model might be a better choice, as the colitic response evolves slowly due to pro-inflammatory responses of T cells in the lymphopenic host. This is of special interest, as Treg cells were shown to be critically involved in carrying the beneficial effect of Av17 treatment in the airway hypereactivity model (Schnoeller et al.; JI 2008). Co-transfer of suboptimal doses of Tregs to SCID mice reconstituted with naïve effector T cells impairs the development of colitis (124) and this model might be of use for the investigation of a presumed Treg-activating effect of Av17. Furthermore, animals with a cell-type restricted IL-10 knockout might provide a useful tool to investigate the role of macrophages and IL-10 with respect to modulation by Av17.

Summing up, this work shows that co-treatment of mice exposed to a colitis-inducing chemical with a nematode immunomodulator significantly inhibits disease development. As similar rates of protection have been ascribed to infection with parasites in similar models, one can

conclude, that the broad spectrum of parasitic helminths is equipped with such immunomodulatory components specifically designed to escape the hosts response against the pathogen. As shown here and elsewhere (Schnoeller et al.; JI 2008), such components might be successfully applied in chronic inflammatory diseases as well as allergic disorders. It would be of clear advantage to have more of such defined components at hand, especially with regard to testing of combined multi-component treatment. It is also clear, that defined proteins or other helminth-derived structures have to be considered as more applicable for disease treatment in comparison to therapeutic worm infections. Although treatment by infections may have a beneficial effect, undesired side effects are probably more threatening compared to treatment with defined parasite components.

Although this work was not able to conclusively address the mechanism of action by filarial cystatin, the work of the group showed that Av17 mediates its anti-allergic effect in an asthma model probably by a combination of IL-10 induction by macrophages and other cells as well as by addressing suppressive cells, namely Treg cells and macrophages. This, together with the notion that all these immune components are discussed to be important for the control of intestinal inflammation, may support the assumption that similar modes of action are responsible for the effect of Av17 on colitis.

Finally, the obvious similarity between the interference of helminth infections with inflammatory models by addressing anti-inflammatory cytokines, regulatory cells and altering APC function, and the proposed targets for cystatin point out that this component might be a precious tool to mimic such alterations.

5 Materials and methods:

5.1 Parasitological methods

5.1.1 Life cycle of *H. polygyrus*

Heligmosomoides polygyrus was maintained by serial passage in BALB/c mice. Mice were infected with approximately 200 L3 using a feeding tube. Infective larvae were obtained from fecal cultures. Feces were collected from infected animals, washed in distilled water and plated in Petri dishes on humid blotting paper for 7 days. Plates were kept in at room temperature with reduced airflow and high humidity provided by a water-filled Petri dish. On day 7, L3 were washed from the plate and kept in distilled water at 4°C after extensive washing. L3 were used for infection up to 8 weeks after fecal culture.

5.1.2 Determination of adult worm burden

Mice in transfer studies were infected with a defined dose of L3. The number of larvae was determined exactly before application. L3 retained in the feeding tube and syringes were subtracted to calculate the exact infection dose. The worm burden was determined by collecting adult worms from the small intestine on the day of dissection. The value was calculated as the percentage of applied L3.

5.2 Colitis model

Female C57/BL6 mice (10-12 weeks old, body weight >20g) were housed at 22°C under controlled SPF conditions. Mice were fed with sterile drinking water containing 2.3 - 2.5% dextran sodium sulfate (DSS, mol. wt 40000, ICN, Eschwege, Germany) for 7 days. Control animals were fed tap water without DSS. 20µg rAv17 or the control protein rDHFR in 200µl buffer was injected intraperitoneally four times over the 7-day DSS feeding period (day 0, 2, 4 and 6). Control animals received DSS and were sham-treated with the protein application buffer. To induce chronic colitis, mice underwent three treatment cycles with DSS (each 5-7 days, depending on monitored weight loss and physical appearance of the animals). Mice

were kept on DSS-free drinking water for at least one week to recover between the DSS cycles. Animals were treated with Av17 or the control protein DHFR according to the aforementioned schedule. A group of mice was left untreated throughout the first DSS-feeding cycle to compare preventive and therapeutic application of Av17. Appearance of feces and weight loss were monitored daily. One day after the last treatment (kept on DSS-free drinking water for approx. 20h), animals were killed by cervical dislocation and the colon was resected between the ileocecal junction and the proximal rectum. The colon was placed on a non-absorbent surface and measured with a ruler. The entire colon was divided into three segments (proximal, middle and distal) and a part of each segment was fixed in 10% neutral buffered formalin. After fixation, specimens were embedded in paraffin, cut into 7 µm sections and stained with hematoxilin and eosin.

The remaining colonic tissue was used for isolation of LPL cells as described in the following.

5.3 Cell culture

5.3.1 Preparation of mesenteric lymph node cells (MLNC), splenocytes, intraepithelial (IEL) and lamina propria (LPL) lymphocytes.

MLN and spleen were isolated aseptically from euthanized mice. Mesenteric lymph node cells (MLNC) and splenocytes were dissociated by passing organs through a steel mesh in PBS pH 7.4 containing 0.2% BSA. Erythrocytes were removed by resuspension of washed cells in lysis buffer for 5 min. on ice. After washing, cells were adjusted to desired concentrations in cRPMI for culture or PBS/BSA for surface stainings and flow cytometric analysis.

Intraepithelial (IEL) and lamina propria (LPL) lymphocytes were isolated from the small intestine (SI). After removing visible Peyer's patches, the SI was opened longitudinally, washed in PBS pH 7.4 and incubated in RPMI at 37°C, 150 rpm for 40 min. The SI was washed twice in PBS and supernatants were collected for isolation of IEL. The organ was cut to pieces and incubated (40-50 min, 37°C, 150 rpm) with collagenase VIII and D (40 µg/ml each, both from Sigma), than tissue was removed with a mesh. Supernatants were spun and the cells layered on a column of Percoll (GE Healthcare, Uppsala, Sweden) with a 40%:70% gradient. Cells were spun at room temperature and 2.200 x g for 20 min, then collected from the interphase, washed and kept in PBS 0.2% BSA.

5.3.2 Generation of bone marrow derived dendritic cells (BmDC)

For generation of DC, bone marrow was isolated from tibia and femur of 6-8 week old naïve BALB/c mice and cells were kept in 24 well plates at a concentration of $1.5x10^6$/ml in cRPMI supplemented with 20 ng/ml of GM-CSF (PeproTech, Hamburg, Germany) for 6 days. On d2, cultures were gently resuspended and medium with detached cells was removed. Fresh cRPMI supplemented with GM-CSF was added. On d4, supernatant was removed and replaced by fresh medium containing GM-CSF. Detached cells were harvested on d6 as immature DC (iDC) and either left untreated (further GM-CSF supplemented culture) or treated with *H. polygyrus* ag.

5.3.3 Cell culture conditions

Cell cultures were performed in cRPMI as triplicates or quadruplicates on 96-well round bottom plates.

5.3.3.1 Suppression assays

Culture of regulatory subsets and $CD4^+CD25^-CD103^-$ responder cells for detection of polyclonal response to αCD3 stimulation (1 µg/ml) was performed with $2.5x10^4$ $CD4^+$ cells and $5x10^4$APC per well. Cells were incubated for 48 h followed by addition of 1 µCi of methyl-[^{3}H]-thymidine per well for 20 h to measure proliferation. In co-incubation assays, the indicated ratios of T_{reg} cells were added to naïve responder cells and APC and treated as described above. Naïve splenocytes depleted of T cells ($CD90^+$) using magnetic beads were used as APC. The $CD90^-$ cell fraction was irradiated (30 Gy) before culture.

5.3.3.2 Culture of complete and T_{reg} cell-depleted MLNC

$3.5x10^5$ cells were incubated in presence of 12µg/ml of adult worm antigen or left untreated for 72 h and supernatants were harvested for cytokine detection. Cultures of cMLNC were compared to MLNC depleted of cells expressing CD25 and/or CD103.

5.3.3.3 Culture of $CD4^+$ T cell subsets with BmDC

Bone marrow derived dendritic cells (BmDC) were generated by a GM-CSF supplemented 6d culture. Immature DC were incubated with 10 µg/ml of adult worm antigen for 12h. Control cells were left untreated. $1x10^4$ DC were cultured for 72 h with $5x10^4$ T cells, then supernatants were harvested for cytokine-ELISA and cells were snap-frozen in liquid nitrogen and stored at –80°C for real-time PCR. Recombinant mouse IL-2 (10 ng/ml; PeproTech, Hamburg, Germany) and 1 µg/ml αCD28 were added to some cultures for optimal stimulation.

5.3.3.4 Culture for detection of Ag-specific CD4$^+$ Teff cells via CD154-expression

For measurement of CD154 (CD40L) expression of T cells and detection of cytokines in antigen-specifically activated cells, splenocytes and MLNC from infected animals were incubated in 24-well plates at a concentration of $4x10^7$ cells per ml with 20 µg/ml of adult worm antigen and 1 µg/ml αCD28 for 12 h. To survey cytokine production, brefeldin A (5 µg/ml, Sigma) was added after the first 2 h of stimulation. For surface staining of CD154, cells were incubated as indicated above but without addition of brefeldin A. αCD154-APC, αFcR II/III mAbs (20 µg/ml), whole rat IgG (10 µg/ml) and αCD40 to avoid rapid removal of CD154 from the cell surface after binding of CD40 expressed on APC) were added to the culture. After 12 hours, cells were washed and prepared for flow cytometric analysis.

5.3.4 Isolation of cell subsets via magnetic beads/FACS

The separation of T cell subsets for transfers and *in vitro* assays was performed as follows: Cells were stained for CD25 (APC) and CD103 (PE). CD25$^+$ and CD103$^+$ cells were enriched by the AutoMACS magnetic separation system using αAPC and αPE magnetic beads. For isolation of the different regulatory subsets, the bead-positive fraction was stained with FITC-labelled αCD4 and the CD4$^+$CD25$^+$CD103$^+$ cells and CD4$^+$CD25$^+$CD103$^-$ cell subsets were separated using a FACS Diva cell sorter (BD, Heidelberg, Germany). After complete removal of cells expressing CD25 and/or CD103, the negative fraction was used to isolate conventional CD4$^+$ T cells using αCD4 beads. Naïve splenocytes depleted of T cells using αCD90 beads were irradiated (30Gy) and used as antigen-presenting cells (APC) for *in vitro* assays. For some adoptive transfers, the whole CD4+CD103$^+$ subset (irrespective of CD25 expression) was isolated. Therefore, cells were stained for CD4 (FITC), CD25 (APC) and CD103 (PE). CD4$^+$

cells were isolated using the FITC-MultiSort kit by AutoMACS. After removal of beads, $CD103^+$ cells were isolated from $CD4^+$ cells using αPE beads.

5.4 *In vivo* adoptive transfers

Sorted $CD4^+$ T cell subpopulations ($5x10^5$ cells per animal) were injected ip into naïve mice in 0.2ml of sterile PBS. Control animals received PBS only. One day after transfer, mice were infected with a defined dose of L3. Four weeks after infection, animals were sacrificed and the adult worm number of each animal was determined and calculated in percent of the exact dose of applied L3 set to 100%. Success of infection was determined by survey of the fecal egg output starting on day 10 pi. To survey cell survival and to trace transferred cells, mice received $1x10^7$ CFSE-labelled or eGFP-expressing $CD4^+$ cells. Reanalysis by flow cytometry was performed six days or 3 weeks after transfer in spleen, MLN and small intestine.

5.5 Immunological methods

5.5.1 Flow cytometric analysis

5.5.1.1 Cell surface staining

Surface staining of lymphocytes using mAb was performed in PBS/BSA on ice for 10 min. For detection of changes in lymphocyte composition, cell suspensions ($1x10^6$ total cells) were stained with αCD4, αCD8 and αCD19 mAb. T_{reg} cells were detected by staining for CD4, CD25 and CD103. Unspecific binding of the mAbs was blocked by the addition of αFcγRII/III (20 µg/ml). Cytometric analysis was performed using FACS Calibur or LSRII (BD Biosciences) and FlowJo software (Tree Star, Inc. Ashland, USA).

5.5.1.2 Intracellular staining

Intracellular detection of Foxp3 was performed according to the manufacturer's instructions. For intracellular detection of CD154 and cytokines, cells were fixed in PBS containing 2% formaldehyde for 15 min at RT. After permeabilization with 0.5% saponin, cells were blocked with whole rat IgG (0.1 mg/ml) for 15 min at 4°C to reduce unspecific binding of mAbs and stained with αCD154 and two of the αmouse cytokine mAbs for 30 min at 4°C. For combined

detection of CD154 and Foxp3, CD154 was stained on the cell surface directly during *in vitro* stimulation in complete culture medium (see above).

5.5.2 Enzyme-linked immunosorbent assay (ELISA)

IL-4, IL-10 and IFN-γ in cell culture supernatants were quantified using OptEIA ELISA-kits (BD Biosciences) according to the manufacturer's instructions. IL-13 and active TGF-β1 was detected using the DuoSets from R&D Systems. Assays were performed according to the manufacturer's instructions, but using 50µl of samples and standards/well. In case of limited sample size, two assays were performed subsequently with the same samples. In this case, IL-4 and IL-10 were the first cytokines to be detected, followed by IFN-γ or IL-13.

TGF-β1 ELISA was performed without pre-activation of samples. Activation led to high background levels due to cRPMI containing 10% FCS.

5.5.3 Activity test of filarial cystatin

After recombinant expression, purification, lipopolysaccheride (LPS) removal and determination of remaining LPS contamination, each charge of rAv17 had to be tested for its biological activity. Therefore, the protein was tested for its for suppressive effect on polyclonal proliferation of mouse splenocytes in response to concanavalin A.

5.5.3.1 Test for in vitro *suppressive capacity*

$3.5x10^5$ splenocytes from a naive BALB/c mouse were stimulated in a volume of 200 µl cRPMI for 48h with 2µg/ml of Con A. Cells were pulsed with ^{3}H-thymidine for 20h and proliferation was detected using a β-counter. Charges showing a suppressive efficiency of at least 20% (Av17 conc*. in vitro* 2.5 to 5 µg/ml) were considered as biological active.

5.5.4 Immunohistological Foxp3-detection in small intestinal cross sections

Immunohistology for Foxp3 expressing cells was performed by C. Loddenkemper at the Charité, Berlin, Germany. Tissue samples from the proximal third of the small intestine of naïve and *H. polygyrus*-infected mice were fixed in 4% phosphate-buffered formalin, embedded in paraffin and used for cross sections. Immunohistology for Foxp3-expressing cells was

performed as described elsewhere (32, 33). Foxp3$^+$ cells were counted in 10 high-power fields (HPF, 40-fold magnification) randomly distributed in sections of each animal (Peyer's patch areas were excluded).

5.6 Molecular biology and biochemical methods

5.6.1 Preparation of *H. polygyrus* adult worm antigen

Soluble worm antigen was prepared from adult worms kept in culture in RPMI medium containing 100 U/ml penicillin and 100 µg/ml streptomycin for 24 h. Worm material was homogenized and sonicated (1 min, 60W) on ice in PBS (pH 7.4). The homogenate was centrifuged (20 min, 20,000*g*, 4°C) and the supernatant was passed through a 0.4 µm filter (Schleicher & Schuell, Germany) for sterilization. The protein content was determined by bicinchoninic acid test (Pierce, USA). Antigen extracts were stored at –80°C until application.

5.6.2 Isolation, purification and reverse-transcription of mRNA

RNA extractions from distinct CD4$^+$ Teff and Treg populations was performed after co-incubation of T cells with naïve or *H. polygyrus*-ag pre-treated bone marrow derived DC were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by digestion of DNA using the RNase-free DNase-Set (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed using the TaqMan Reverse Transcription Reagent (Applied Biosystems, Warrington, UK) and oligo dTs.

5.6.3 Quantitative PCR

Quantitative real-time PCR for transcript quantification of IL-4 and IL-10 was performed with the 7300 Real-Time PCR System (Applied Biosystems) using TaqMan reagents (Applied Biosystems). PCR amplifications were done in triplicates containing 3 µl of cDNA, 2 µl of 20x TaqMan labelled primer mix and 10 µl of 2x TaqMan PCR buffer. The 20x TaqMan primer mix consisted of two unlabeled PCR primers (900 nM each final concentration) and 1FAM™ dye-labeled TaqMan®MGB probe (250 nM final concentration). All primers were obtained from Applied Biosystems (IL-4 Assay ID: Mm00445259_m1, IL-10 Assay ID: Mm00439616_m1, GAPDH Assay ID: Mm99999915_g1). Real-time PCR was performed using the following condi-

tions: 10 min denaturation at 95°C followed by 40 amplification cycles of 15 s at 95°C and 60 s at 60°C. The relative amounts of IL-10 and IL-4 mRNA were normalized to the endogenous reference GAPDH. Quantification of transcripts in cells cultured in the presence of DC pre-treated with H. polygyrus antigen was done relative to cells cultured with naïve DC using the $2^{-\Delta\Delta Ct}$ method as described in (31).

5.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, USA). Statistical significance as indicated in the figure legends was either analyzed by Mann-Whitney test or ANOVA in combination with Bonferroni post-tests.

5.8 Material

5.8.1 Animals used for experiments

BALB/c and C57BL/6 mice were purchased from the BfR, Berlin. C57BL/6-Tg(CAG-EGFP)C15-001-FJ001Osb mice were a gift of M. Okawa, Osaka University. Animals were housed and handled following national guidelines and as approved by our animal ethics committee.

5.8.2 Laboratory equipment

Flow cytometer (analysis):	Calibur; LSRII, BD Biosystems, San Jose, CA
Flow cytometer (sorting):	FACSDiva; FACSAria, BD Biosystems, San Jose, CA
AutoMACS:	Miltenyi, Bergisch-Gladbach, Germany
ELISA-reader:	Dynatech, Denkendorf, Germany
Scintillation-spectroscope:	Trilux 1450, Wallac, Turku, Finland
Ultrasound-disintegrator:	Heinemann, Schw. Gmünd, Germany
Real-time PCR system 7300:	Applied Biosystems, Foster City, CA

5.8.3 Buffers and media

FACS staining buffer	PBS / 0.2% BSA
FACS fixation buffer	PBS / 0.2% BSA / 2% paraformaldehyde
Permeabilization buffer	PBS / 0.2% BSA / 0.5% saponin
Blocking buffer for intracellular staining	PBS / 0.2% BSA / 0.5% saponin / 100µg/ml polyclonal

	rat IgG
Erythrocyte lysis buffer	0.01M $KHCO_3$
	0.155M NH_4Cl
	0.1mM EDTA
	pH: 7.5
Cell culture medium (cRPMI)	RPMI-1640
	10% FCS
	20 mM L-glutamine
	100 U/ml penicillin
	100 µg/ml streptomycin
	all from Biochrom, Berlin, Germany
Digestion medium for LPL isolation	cRPMI
	40µg/ml collagenase D
	40 µg/ml collagenase VIII

5.8.4 Chemicals, biologicals and recombinant cytokines

Saponin	Sigma, Munich, Germany
Caboxyfluoroscein succinimidyl ester (CFSE)	Sigma, Munich, Germany
Percoll	GE Healthcare, Chalfont St. Giles, Great Britain
3H Thymidin	ICN, Costa Mesa, USA
Brefeldin A	Sigma, München, Germany
PMA	Sigma, München, Germany
GM-CSF/IL-2	PeproTech, Hamburg, Germany
BSA, fraction V	AppliChem, Darmstadt, Germany
Proteinase inhibitor cocktail	Roche, Mannheim, Germany
Paraformaldehyde	Sigma, München, Germany
Collagenase D	Roche, Mannheim, Germany
Collagenase VIII	Sigma, München, Germany

5.8.5 Kits

Foxp3 detection kit, (clone FJK-16s, PE-coupled)	eBiosciences, San Diego, CA
OptEIA ELISA kits	BD Biosciences, Heidelberg, Germany
IL-13 and TGF-b ELISA-sets	R&D Systems, Minneapolis, MN
BCA kit	Pierce, USA
TaqMan primer and probes	Applied Biosystems, Darmstadt, Germany

TaqMan cDNA synthesis kit	Applied Biosystems, Germany
TaqMan PCR master mix	Applied Biosystems, Germany
RNeasy Mini kit	Qiagen, Hilden, Germany
QIAshredder spin columns	Qiagen, Hilden, Germany
LAL QCL-1000	Cambrex, USA

5.8.6 Magnetic beads

The following MicroBeads were obtained from from Miltenyi Biotec (Bergisch-Gladbach, Germany):

αCD4, αCD90, αPE, αAPC, αFITC MultiSort kit

5.8.7 Antibodies and secondary reagents

Specificity	fluorochrome	clone	Purchased from
αCD3e	-	145-2C11	BD Biosciences
αCD4	FITC/PerCP	RM4-5	BD Biosciences
αCD8	PE	53-6.7	BD Biosciences
αCD19	FITC	ID3	DRFZ**
αCD25	APC/PerCP-Cy5.5	PC61	BD Biosciences
αCD28	-	37.51	BD Biosciences
αCD40	-	HM40-3	BD Biosciences
αCD103	PE	M290	BD Biosciences
αCD103	- (biotin)	M290	DRFZ**
αCD154 (CD40L)	APC	MR1	Miltenyi Biotec
αIL-4	FITC	11B11	BD Biosciences
αIL-10	PE	JES5-16E3	BD Biosciences
αIL-13*	-	38213.11	R&D Systems
αIFN-γ	FITC	XMG1.2	BD Biosciences
αFcR II/III	-	2.4G2	DRFZ**
αdigoxigenin	PE		DRFZ**
Whole rat IgG	-	polyclonal	Dianova

Secondary reagents

SA-PE-Cy7	PECy7		BD Biosciences
SA-PE	PE		BD Biosciences

* coupled to digoxigenin in the DRFZ (German Arthritis Research Center, Berlin, Germany).

** mAb was a kind gift of the German Arthritis Research Center .

5.8.8 Software

FloJo (Tristar)

BD FACSDiva (BD Biosciences)

CellQuest (BD Biosciences)

Prism (GraphPad)

6 Appendix: Tregs and pathogens

Pathogen	Cell type	Ag-spec.	Cytok. profile	Suppressed responses	Manipulation of regulatory cells	Effect on imm. response, pathology and pathogen load	Ref.
Bacteria							
Helicobacter hepaticus	Mouse $CD45RB^{low}$ T cell	Yes	IL-10	HpAg-spec. suppr. of IFN-γ production by IL-$10^{-/-}$ $CD4^+$ cells	Transfer of $CD45RB^{low}$ cells	Prevents colitis in IL-$10^{-/-}$ mice	117
	Mouse $CD45RB^{low}$ $CD25^+$	ND	ND	ND	Transfer of $CD45RB^{low}$ $CD25^+$ cells to $RAG^{-/-}$	Intest. inflam. ⇓ innate imm. Resp. ⇓ PL. ⇔	141
Helicobacter pylori	Mouse $CD4^+CD25^+$	ND	ND	ND	Reconstitution of Nu/nu mice with $CD25^-$ LN cells	PL ⇑ Gastritis ⇑ Cellular immune response to Hp-Ag	181
Mycobacterium tuberculosis	Human T_R1 cell	Yes	IL-10	Allogenic $CD4^+$ T cell proliferation	ND	ND	47
	Human $CD4^+CD25^+$	Yes	ND	Ag-spec. IFN-γ and IL-10 production	Depeltion of Treg *in vitro*	Increased response against MT Ag	36
	Mouse $CD4^+CD25^+$	ND	ND	ND	Transfer to $RAG^{-/-}$ mice	Prevents bacterial clearance and IFN-γ by Th1 in $RAG^{-/-}$	118
Protozoa							
Leishmania major	Mouse (C57BL/6) $CD4^+CD25^+$	Yes	IL-10	$CD4^+$ T cell prolif. Ag-spec. IFN-γ production	Transfer to $RAG^{-/-}$, IL-$10^{-/-}$and ic mice	Delayed parasite clearance, lesion size ⇑,PL ⇑	23
	Mouse (BALB/c) $CD4^+CD25^+$	ND	ND	ND	[a]Transfer of $CD25^-$ splenocytes to SCID [b]$CD25^+$ inactvation	[a]failure to control parasite, IL-4 ⇑ [b]lesion size ⇑ PL ⇑	8
	Mouse (C57BL/6) $CD4^+CD25^+$	ND	ND	ND	[a]Transfer to chron. inf. ic mice [b]$CD25^+$ inactivation	[a]Disease react., IFN-γ/IL-10 ⇑ [b]par. control IFN-γ ⇑	149
	Mouse (C57BL/6) $CD4^+CD25^+$	Yes	IL-10	cytokine production by $CD4^+CD25^-$	Transfer of Ag-spec. $Foxp3^+$ T cell lines to IC mice	Failure to control parasitaemia, PL ⇑	215
	Mouse (BALB/c) $CD4^+CD25^+$	ND	ND	ND	Transfer of $CD25^-$ splenocytes to SCID mice	Ag-spec. IFN-γ/IL-4 ⇑, PL, colon lesions ⇑	252
Leishmania amazonensis	Mouse $CD4^+CD25^+$	Yes	IL-10	$CD4^+$ T cell prolif. Ag-spec. IL-2/IFN-γ production	[a]Transfer to IC mice [b]Transfer of $CD25^-$ splenocytes to $RAG^{-/-}$	[a]Lesion size ⇓ PL ⇓,IL-4/ IFN-γ ⇓ [b]susceptibility⇑	106
Leishmania braziliensis	Human $CD4^+CD25^+$	ND	IL-10 / TGF-β	$CD4^+$ T cell proliferation	ND	ND	30
Leishmania donovani	Human $CD4^+CD25^+$	ND	ND	ND	$CD25^+$ Depletion *in vitro*	PBMC IFN-γ resp. ⇔	164
Plasmodium chabaudi adami	Mouse $CD4^+CD25^+$	ND	ND	ND	CD25 depletion *in vivo*	PL ⇑ (lethal strain) PL ⇔ (non-l. strain) Cytokine prod. ⇑	29

Plasmodium berghei	Mouse $CD4^+CD25^+$	ND	ND	ND	$CD25^+$ depletion/inact. *in vitro* and *in vivo*	Ag-spec. prolif. / IL-2 ⇑, IL-10 ⇓1° inf.: PL ⇔ fatality ⇔ Th1 memory ⇓2° inf.: PL ⇓, pathology ⇑	162
Plasmodium falciparum	Human $CD4^+CD25^+$	ND	ND	PBMC proliferation, IFN-γ response	PBMC $CD25^+$depletion *in vitro*	IFN-γ ⇑ Proliferation ⇑	254
Plasmodium yoelii	Mouse $CD4^+CD25^+$	ND	ND	ND	$CD25^+$ inactivation	Restores prolif. Control of PL Non-lethal outcome	94
Trypanosoma cruzi	Mouse $CD4^+CD25^+$	ND	ND	ND	$CD25^+$ inactivation *in vivo*	$CD8^+$ expansion/ IFN-γ response/ survival rate⇔	116
Helminths							
Onchocerca volvulus	Human Th3 and T_R1 cell	Yes	IL-10 and/or TGF-β	PBMC proliferation	ND	ND	51, 193
Pathogen	Cell type	Ag-spec.	Cytok. profile	Suppressed responses	Manipulation of regulatory cells	Effect on imm. response, pathology and pathogen load	Ref.
Litosomoides sigmodontis	Mouse $CD4^+CD25^+$	ND	ND	Fail to suppress $CD25^-$ proliferation	$CD25^+$ inactivation *in vivo* + α-GITR-treatment	Red. worm burden Restoration of Th2/IL-10 response	227
	Mouse $CD4^+CD25^+$	ND	ND	ND	$CD25^+$ inact. *in vivo* + α-CTLA-4-treatment	Reduction of worm burden	225
Brugia pahangi	Mouse $CD4^+CD25^+$	ND	IL-10	ND	$CD25^+$ inactivation *in vivo*	Increased Ag-spec. proliferation/Th2 response	77
Heligmosomoides polygyrus	Mouse $CD4^+CD25^+$	ND	ND	ND	Transfer	Protective in asthma model	250
	Mouse $CD3^+$	ND	ND	ND	Transfer of MLN cells with increased Foxp3 expression	$IL\text{-}10^{-/-}$ T cells from Hp-inf. mice transfer protection against colitis to $IL\text{-}10^{-/-}$ mice	56
	Mouse $CD8^+$ lamina propria T cells	ND	ND	T cell proliferation (IL-10 and TGF-β indep.)	Transfer to $RAG^{-/-}$ mice	Protection against colitis	150
	Lamina propria $CD4^+$ T cells	ND	IL-10	T cell INF-γ production	Transfer to wild type mice	Il-10 dep. rotection from chemically induced colitis	201
	Mouse $CD4^+CD25^+$	ND	ND	$CD4^+$ T cell proliferation	ND	ND	68
	Mouse $CD4^+CD25^+$	Yes	IL-10	$CD4^+$ T cell proliferation Ag-spec IL-4 prod.	Transfer to IC mice	No effect on PL	IAI Vol. 76; 5 2008
Ascaris suum	Mouse $CD4^+CD25^+$	ND	ND	ND	Transfer to allergen challenged mice	Suppression of conjunctivitis	196
Trichinella spiralis	Mouse $CD4^+CD25^+$	ND	ND	ND	[a] $CD25^+$ inactivation *in vivo*	[a]Th1/Th2/IL-10 resp. ⇑	19

					[b]Transfer to RAG/IL-10$^{-/-}$	muscle larvae burden ⇔ [b]minor T_{reg} role in contr. IFN-γ resp.	
Schistosoma mansoni	Mouse $CD4^+CD25^+$	ND	IL-10 (low)	$CD4^+$ T cell proliferation	Transfer of complete / $CD25^+$-depleted $CD4^+$ cells to RAG$^{-/-}$ or RAG/IL-10$^{-/-}$	T_{reg} control pathology and increase survival rate	92
	Mouse $CD4^+CD25^+$/ $CD4^+CD25^-$	Yes	IL-10	$CD4^+$ T cell prolif. IL-12 prod. by DC	Transfer to IL-10$^{-/-}$ mice	Reduced Th1 resp. to egg Ag	146
	Mouse $CD4^+CD25^+$	ND	IL-10 (low)	ND	$CD25^+$ inact. *in vivo*	Increased IL-4 response	15
	Mouse $CD4^+CD25^+$	Yes	IL-10	ND	$CD25^+$ inact. *in vivo*	Increased Th1/Th2 response	224
	Mouse $CD4^+CD25^+$	Yes	IL-10 (low)	Ag-spec. $CD4^+$ T cell proliferation IL-10 production by $CD25^-$ T cells	Transfer to TLR-2$^{-/-}$ or wt mice	In TLR-2$^{-/-}$: granuloma size/IFN-γ resp.⇓ Liver egg burden⇑	122
	Mouse $CD4^+CD25^+$	ND	ND	ND	$CD25^+$ inactivation *in vivo*	Weight ⇔ Cytokine resp.⇔ Par. fecundity ⇔ pathology ⇔	244
Schistosoma japonicum	Mouse $CD4^+CD25^+$	Yes	IL-10	Schist. egg- ind. $CD25^+$ Tregs supp. Ag-spec. $CD4^+$ T cell proliferation	$CD25^+$ inactivation *in vivo*	Reverses protective effect of schist. eggs on asthma	253

Index: ND: not done; 1°: primary; 2° secondary, PL: parasite load; IC: immunocompetent; PBMC: peripheral blood mononuclear cells; RAG: recombination-activating gene; SCID: severe combined immunodeficiency; TLR-2: Toll-like receptor 2.

7 Abbreviations

1°	primary infection
2°	secondary infection
Ab	antibody
Ag	antigen
APC	antigen presenting cell
APC	allophycocyanin
Av17	recombinant *Acanthocheilonema viteae* cystatin (mol. weight 17 kD)
Bm	bone marrow
BmDC	bone marrow derived dendritic cell
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	carboxyfluoroscein succinimidyl ester
CTLA-4	
DC	dendritic cell
DHFR	dihydrofolate reductase
DSS	dextran sodium sulphate
dpi	days post infection
(e)GFP	(enhanced) green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box transcription factor p3
FSC	forward scatter
g	G force
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
ic	intracellular
IC	immunocompetent
ID	immunodeficient
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immune globulin
IL	interleukin

KO	knock out
LPL	lamina propria lymphocyte
M	molar
mAb	monoclonal antibody
min	minute
MLN	mesenteric lymph node
MLNC	mesenteric lymph node cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pi	post infection
PE	phycoerythrin
PLN	peripheral lymph node
r	recombinant
SA	streptavidin
SI	small intestine
Teff	effector T cell
TGF-β	transforming growth factor beta
Treg	regulatory T cell
1°	primary infection
2°	secondary infection
Ab	antibody
Ag	antigen
APC	antigen presenting cell
APC	allophycocyanin
Av17	recombinant *Acanthocheilonema viteae* cystatin (mol. weight 17 kD)
Bm	bone marrow
BmDC	bone marrow derived dendritic cell
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	carboxyfluoroscein succinimidyl ester
CTLA-4	
DC	dendritic cell
DHFR	dihydrofolate reductase
DSS	dextran sodium sulphate
dpi	days post infection
(e)GFP	(enhanced) green fluorescent protein
ELISA	enzyme-linked immunosorbent assay

FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box transcription factor p3
FSC	forward scatter
g	G force
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
ic	intracellular
IC	immunocompetent
ID	immunodeficient
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immune globulin
IL	interleukin
KO	knock out
LPL	lamina propria lymphocyte
M	molar
mAb	monoclonal antibody
min	minute
MLN	mesenteric lymph node
MLNC	mesenteric lymph node cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pi	post infection
PE	phycoerythrin
PLN	peripheral lymph node
r	recombinant
SA	streptavidin
SI	small intestine
Teff	effector T cell
TGF-β	transforming growth factor beta
Treg	regulatory T cell
1°	primary infection
2°	secondary infection
Ab	antibody

Ag	antigen
APC	antigen presenting cell
APC	allophycocyanin
Av17	recombinant *Acanthocheilonema viteae* cystatin (mol. weight 17 kD)
Bm	bone marrow
BmDC	bone marrow derived dendritic cell
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	carboxyfluoroscein succinimidyl ester
CTLA-4	
DC	dendritic cell
DHFR	dihydrofolate reductase
DSS	dextran sodium sulphate
dpi	days post infection
(e)GFP	(enhanced) green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box transcription factor p3
FSC	forward scatter
g	G force
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
ic	intracellular
IC	immunocompetent

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7 Publications and Presentations

Publications

S. Rausch, J. Huehn, D. Kirchhoff, J. Rzepecka, C. Schnoeller, S. Pillai, C. Loddenkemper, A. Scheffold, A. Hamann, R. Lucius, and S. Hartmann, *Functional Analysis of Effector and Regulatory T cells in a Parasitic Nematode Infection,* Infection And Immunity, Vol. 76, 2008.

Schnoeller C, **Rausch S**, Pillai S, Avagyan A, Wittig BM, Loddenkemper C, Hamann A, Hamelmann E, Lucius R, Hartmann S., *A Helminth Immunomodulator Reduces Allergic and Inflammatory Responses by Induction of IL-10-Producing Macrophages,* Journal of Immunology, Vol. 180, 2008.

Kirchhoff D, Frentsch M, Leclerk P, Bumann D, **Rausch S**, Hartmann S, Thiel A, Scheffold A. *Identification and Isolation of Murine Antigen-reactive T Cells According to CD154 Expression,* European Journal of Immunology, Vol. 37, 2007.

Rzepecka J, Lucius R, Doligalska M, Beck S, **Rausch S**, Hartmann S., *Screening for Immunomodulatory Proteins of the Intestinal Parasitic Nematode* Heligmosomoides polygyrus*.,* Parasite Immunology, Vol. 28, 2006.

Bruni N, Knebel T, Bongartz J, **Rausch S**, Hochreiter S, Volkmer R, Or-Guil M. *Random Generated Peptide Libraries for Classification and Prediction of Serum Antibody Patterns,* International Journal of Bioinformatics (accepted)

J. Rzepecka, R. Lucius, **S. Rausch**, C. Schnöller, J. Hagen, R. Ignatius and S. Hartmann, *Functional Characterization of Calreticulin from the Intestinal Nematode* Heligmosomoides polygyrus*.* (In preparation)

S. Hartmann, C. Schnöller, A. Dahten, A. Avagyan, **S. Rausch**, M. Lendner, S. Pillai, C. Bocian, R. Lucius, M. Worm, E. Hamelmann, *Gastrointestinal Nematode Infection Inhibits Experimental Allergic Airway Inflammation but not Atopic Dermatitis,* (In preparation)

Bruni N, Knebel T, Bongartz J, **Rausch S**, Hochreiter S, Schutkowski M, Or-Guil M, *Classification of Whole Serum Antibody Reactivities with Random Peptide Libraries,* (In preparation*)*

Conference abstracts

S. Rausch, J. Hühn, B. Sonnenburg, R. Lucius, S. Hartmann, Murine regulatory T cell response to an intestinal nematode infection, 20th Congress of the Polish Parasitological SocietySeptember 2004, Warsaw, Poland (Talk)

S. Rausch, J. Hühn, B. Sonnenburg, R. Lucius, S. Hartmann, Functional Analysis of regulatory T cells in mice infected with *Heligmosomoides polygyrus,* Spring meeting of the British Society for Parasitology, April 2005, Nottingham, Great Britain, (Talk)

S.Rausch, R. Lucius, S. Hartmann, Immunomodulation by parasitic nematodes, Retreat of the SFB 650, June 2005, Berlin, Germany (Talk)

S. Rausch, J. Hühn, B. Sonnenburg, C. Siewert, R. Lucius, S. Hartmann. Functional analysis of regulatory T cells in mice infected with the intestinal nematode *Heligmosomoides polygyrus.* Joint Annual meeting of the German and Scandinavian Societies for Immunology, September 2005, Kiel, Germany (Poster)

S. Rausch, J. Hühn, J. Rzepecka, B. Sonnenburg, R. Lucius, S. Hartmann, Functional analysis of regulatory T cells in mice infected with the intestinal nematode *Heligmosomoides polygyrus,* Woods Hole ImmunoParasitology Meeting, April 2006, Woods Hole, USA (Poster)

S. Rausch, J. Hühn, J. Rzepecka, B. Sonnenburg, R. Lucius, S. Hartmann, Functional analysis of regulatory T cells in mice infected with the intestinal nematode *Heligmosomoides polygyrus.* Annual Meeting of the German Society for Parasitology, February 2006, Wien, Austria, (Talk)

S. Rausch, J. Hühn, D. Kirchhoff, J. Rzepecka, C. Schnöller, S. Pillai, C. Loddenkemper, A. Hamann, R. Lucius, S. Hartmann. Functional analysis of effector and regulatory T cells in a parasitic nematode infection, Annual Meeting of the German Society for Parasitology, March 2008, Hamburg, Germany (Talk)

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben.

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